2007

Glutamate Excitotoxicity In Epilepsy And Ischemia

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GLUTAMATE EXCITOTOXICITY IN EPILEPSY AND ISCHEMIA

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida,
Orlando, Florida

Fall term
2007

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ABSTRACT

'Excitotoxicity' represents the excitatory amino acid mediated degeneration of neurons. Glutamate is the major excitatory neurotransmitter in the brain. Glutamate excitotoxicity has been implicated in a number of neurodegenerative disorders like Stroke, Epilepsy, Alzheimer's disease and traumatic brain injury. This neurotoxicity is summed up by the 'glutamate hypothesis' which describes the cause of neuronal cell death as an excessive release of glutamate causing over excitation of the glutamate receptors and subsequent increase in influx of calcium leading to cell death. An effort to counteract this neurotoxicity has lead to the development of glutamate receptor antagonists that can effectively serve as neuroprotective agents. Nevertheless, the downside to these drugs has been the side effects observed in clinical trial patients due to their disruptive action on the physiological function of these receptors like learning and memory.

This work was undertaken to identify targets that can effectively be used to treat excitotoxicity without affecting any normal physiological functions. In one approach, (chapter I) we have identified the K_{ATP} channels as an effective modulator of epileptogenesis. In another approach, (Chapter II) we show that targeting the AMPA receptor subunit GluR2 is a practical strategy for stroke therapy.

K_{ATP} channels that are gated by intracellular ATP/ADP concentrations are a unique subtype of potassium channels and play an essential role in coupling intracellular metabolic events to electrical activity. Opening of K_{ATP} channels during energy deficits in the central nervous system (CNS) induces efflux of potassium ions and in turn hyperpolarizes neurons. Thus, activation of K_{ATP} channels is thought to be able to counteract excitatory insults and protect against neuronal death. Here, we show that, functional Kir6.1 channels are located at

excitatory pre-synaptic terminals as a complex with type-1 Sulfonylurea receptors (SUR1) in the hippocampus. The mutant mice with deficiencies in expressing the Kir6.1 or the SUR1 gene are more vulnerable to generation of epileptic form of seizures, compared to wild-type controls. Whole-cell patch clamp recordings demonstrate that genetic deletion of the Kir6.1/SUR1 channels enhances glutamate release at CA3 synapses. Hence, expression of functional Kir6.1/SUR1 channels inhibits seizure responses and possibly acts via limiting excitatory glutamate release.

In addition to epilepsy, ischemic stroke is a leading cause of death in developed countries. A critical feature of this disease is a highly selective pattern of neuronal loss; certain identifiable subsets of neurons, particularly CA1 pyramidal neurons in the hippocampus are severely damaged, whereas others remain intact. A key step in this selective neuronal injury is Ca^{2+}/Zn^{2+} entry into vulnerable neurons through α-amino-*3*-hydroxy-*5*-methyl-*4*-isoxazole propionic acid (AMPA) receptor channels, a principle subtype of glutamate receptors. AMPA receptor channels are assembled from glutamate receptor (GluR) -1, -2, -3, and -4 subunits. Circumstance data have indicated that the GluR2 subunits dictate Ca^{2+}/Zn^{2+} permeability of AMPA receptor channels and gate injurious Ca^{2+}/Zn^{2+} signals in vulnerable neurons. Here we show that ischemic insults induce toxic Ca^{2+} entry through AMPA receptors into vulnerable neurons by modification of GluR2 RNA editing. Thus, targeting of GluR2 subunit can be considered as a promising target for stroke therapy.

Dedicated to

my amma and appa

Without their love, support and nurture this amazing process would have been impossible. Their role in my life has been a tremendous influence on all my accomplishments. I carry the values and strength I draw from them as I jump over every fence.

ACKNOWLEDGMENTS

My foremost thanks to my advisor, Dr YouMing Lu. Without his guidance, support and unbelievable patience my PhD would not have been possible. He has been there every single time I needed him. His mentoring has shown me my strengths, corrected my imperfections and has helped me develop as a scientist. I thank him for showing me the fun in doing science and for teaching me, happiness is indeed possible in science. Chief, you have been an inspiration and I hope one of these days I measure up to you. My research was supported by the NIH/NINDS grants RO1NS051383 and AHA grants 69018001, 69018006 to Dr Lu.

Thanks to my committee members Dr Pappachan Kolattukudy, Dr Steven Chan and Dr Ella Bossy-Wetzel for their constructive criticisms. I especially thank Dr Chan and Dr Bossy-Wetzel for playing a special part in my scientific development through the journal clubs and the discussions during the course of my PhD.

I sincerely thank my lab colleagues Lisheng Peng, for being my friend and answering my million questions. Xiaofen Zhong, for all the hardship, it took her to make me understand electrophysiology and for trying to teach me Chinese at which she failed miserably. Their camaraderie has showed me the importance and value of having an approachable senior in the lab. I thank WeiHong Tu and Wu Di for their help and encouragement. I thank all of them for accepting me, tolerating my craziness and doing their best everyday to make me feel I am one of them.

I thank Dr Tony Zervos for taking me in and guiding me through my baby steps when I was so far away from everything that was familiar. Thanks to Lucia Cilenti for being a friend and for her patience when I thought “minnie” prep was a Disney character. The time I spent at the

Zervos lab was essential for my realization for what I really want to do in science. My special thanks to Dr Laurence Von Kalm, who served as a member in my committee. His support and fairness has helped me through the toughest times of my PhD. My thanks to Dr William Self and Dr Annette Khaled for being in my first committee.

Throughout this process at UCF, exceptional people have pushed me forward and encouraged me in so many ways. My friends, Arul Sahadevan and Raja Ganesan for the endless hours they listened to my complaints and the endless times they told me it will be all right. I thank them for helping me keep my belief in this process; Nitya Venkataraman, for the various ways she brought home so close; Valerie Stratico, who was my "go to girl" in my efforts to adjust to strange America. Matching her "expertise" in the lab and dressing up with her for Halloween has been some of the happiest times of my PhD; Todd Castoe, who in his "infinite beer-derived wisdom" knew the right things to say every time to make me bounce back. I will always miss the countless hours I have spent around his table shedding blood over our course work. Above all, I thank him for surviving and showing me how insignificant a failed experiment was. I thank, Pamela Bagsiyao for her support, the countless runs, lunches and small talk that helped me come back to deal with the every day chaos; Gero Nootz, who was late for the party, but stuck with me to the very end and helped me with so many things personal and academic. I thank, Roman Nemec, for being there for me, for teaching me that determination helps survive the toughest times and for the countless times he fixed the cars I crashed. His remarkable come back has helped me to develop a greater appreciation for my work and to realize it is worth the pain; Pierre Decayette, who dreaded my phone calls but answered every one of them. I thank my friends, Mahua Chaudhury, Raja Kanagaraj, Sarath Dhananjayan, Priya

Ramamoorthy, Max Chen, Claudia Romero, David Moe and Anita Ellappan for helping to pull me through. I thank these people for their loyalty, passion and presence whenever I needed them.

I thank Ms. Kitty and Ms. Sue for the music they have brought into my life that changed my everyday despair into everyday success. I thank my instructors at Butokukan UCF, Mr. Gary Cox, Mr. Stanley Wreblewski, Mr. Rick Vallaro, Rusty Fadgen, Rory Mulcahy and the number of other fellow students whose skills and friendship has enriched my experience at UCF.

It is very difficult to thank my family enough for all they have been to me through out my life and especially this tedious process. I thank my uncle S.P. Swamy for his belief in me and being my model for success; my uncle Jayaseelan Krishnamoorthy for his trust, encouragement and constantly reminding me that I can do this; my brother Suresh Raman, who has been my leaning pole all through these years and for trouble shooting my life every single day. I owe so much to his love, advice and moral support. My aspiration to improve is largely my constant effort to catch up with him; my brother Siva Swamy, one of the most incredible people in my life, for trusting that somebody who comes up with "calcometer" could get her PhD. I am indebted to him for initiating a process that has changed me in so many aspects. I thank him for being there since my "full-time GRE student days" to "my paper is published days"; my little sister, my best friend, Shenbaga Soundarapandian for all the times she cheered me on, for her confidence in me. I thank her for dealing with the realities of life on my behalf so I can do what I want to do in blissful ignorance. Her attitude, strength and character have been a huge reason for my success. I thank my father, Soundarapandian Shanmugam and my mother, Subbulakshmi Subramaniam for the innumerable sacrifices they have made, and for the anxiety and pain, they have gone through to make it possible for me to complete this process. I thank them for their

support, for their recognition of my goals, their love and care. For this, I dedicate this thesis to them. To me, being their daughter is an honor higher than any degree could ever offer.

The most difficult job is to thank the people who are on your cell phone speed dial. One such person is my friend, counselor and the voluntary chair of the "let Mangala graduate committee" Kathleen Nemec. I cannot thank her enough for her trust, love and relentless support that have been my principal strength through out my PhD. I thank her for the countless pep talk lunches that pushed me to finish this process. I thank her, for her trust in me and for constantly improving my confidence and character. I thank her, for taking me into her family and giving me a home so far from home. I thank her, for all the times that she "mommies" me, for all the force feeding and everything that she wont stop doing for me as long as I shall live….

Finally, my thanks to George Kyriazis, for everything, for being my best friend, for making my reason stronger, for being the shoulder to cry on, for listening and preaching. I thank him for catching me on my every downfall, above all seeing me for what I am and showing me what I could become. I thank him for his friendship that I have come to count on more than my own abilities. He being no farther than a phone call or the end of the hallway is why I have survived this frustrating process. I cannot even begin to appreciate the chitchats, the Ecco Domani, the coffee breaks, the arguments and the hard rock concerts. I am glad that "I owe you without having to repay you" for I do not see how. I thank you for suffering this with me.

All these people and many more are the reason I stand here today. I could not have gone through this process without them. For this, I thank them. Above all, I thank science for letting me be a part of the truth. Thank you.

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Back Ground

Epilepsy

Epilepsy is a neurological disease arising from severe seizure activity in the brain. It is a high-risk disease affecting 24 to 53 people per 100000 individuals in developed countries [1, 2]. About 70% of the cases are idiopathic, meaning they have no clear underlying cause. The rest of the causes, range from genetic factors to head trauma or stroke. Some of the types of epilepsy are childhood absence epilepsy, benign focal epilepsy, juvenile myoclonic epilepsy and temporal lobe epilepsy (TLE) [3]. TLE is the most common form of epilepsy in adults. It arises due to seizure activity in the temporal lobes. It is further classified based on origin into Mesial TLE arising from the hippocampus, amygdala or the para hippocampal gyrus and Lateral TLE arising from the neocortex. Chronic TLE results in hippocampal sclerosis causing memory problems in adults and learning disabilities in children. It commonly manifests in a status epilepticus state or recurrent seizures, which might lead to brain damage. It is also very resistant to drugs and often requires surgical intervention. Although extensive research has been done using animal models to characterize this form of epilepsy, there are still huge gaps to be filled before an effective strategy is formed to combat this disease [4-7].

Neuronal activity is a balance between the excitatory and inhibitory signals from the presynaptic termini. Thus, the uncontrolled firing during epileptogenesis is a result of decreased

GABA release from the pre-synaptic terminals and hence decreased inhibition or increased excitatory glutamate release leading to increased excitation and eventually excitotoxicity [8-11] .

Excitatory glutamate synaptic transmission and neuronal degeneration

Glutamate is the major excitatory transmitter in the mammalian central nervous system (CNS) and plays an essential role in neural development, excitatory synaptic transmission, and plasticity [12]. On the other hand, glutamate mediated excitatory insults are implicated in a number of acute neurological disorders including stroke [13, 14] and traumatic brain injury [15] as well as in chronic neurological disorders such as multiple sclerosis, Huntington's disease, Parkinson's disease and Alzheimer's disease [16]. In case of energy deficits (i.e., reduction of intracellular ATP concentration) however, glutamate accumulates in the synaptic cleft due to increased glutamate release from pre-synaptic termini and decreased glutamate uptake by the astrocytes [17, 18]. This results in extensive stimulation of the glutamate receptors and is eventually toxic to the neurons [12, 19].

Glutamate activates three classes of ionophore-linked postsynaptic receptors, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors [20, 21]. Activation and opening of these channels causes an influx of cations and opening of Ca^{2+} channels [22]. The subsequent influx of Ca^{2+} causes further glutamate release and initiates the process of neuronal cell loss. This phenomenon has been referred to as excitotoxicity (Figure 1) [23].

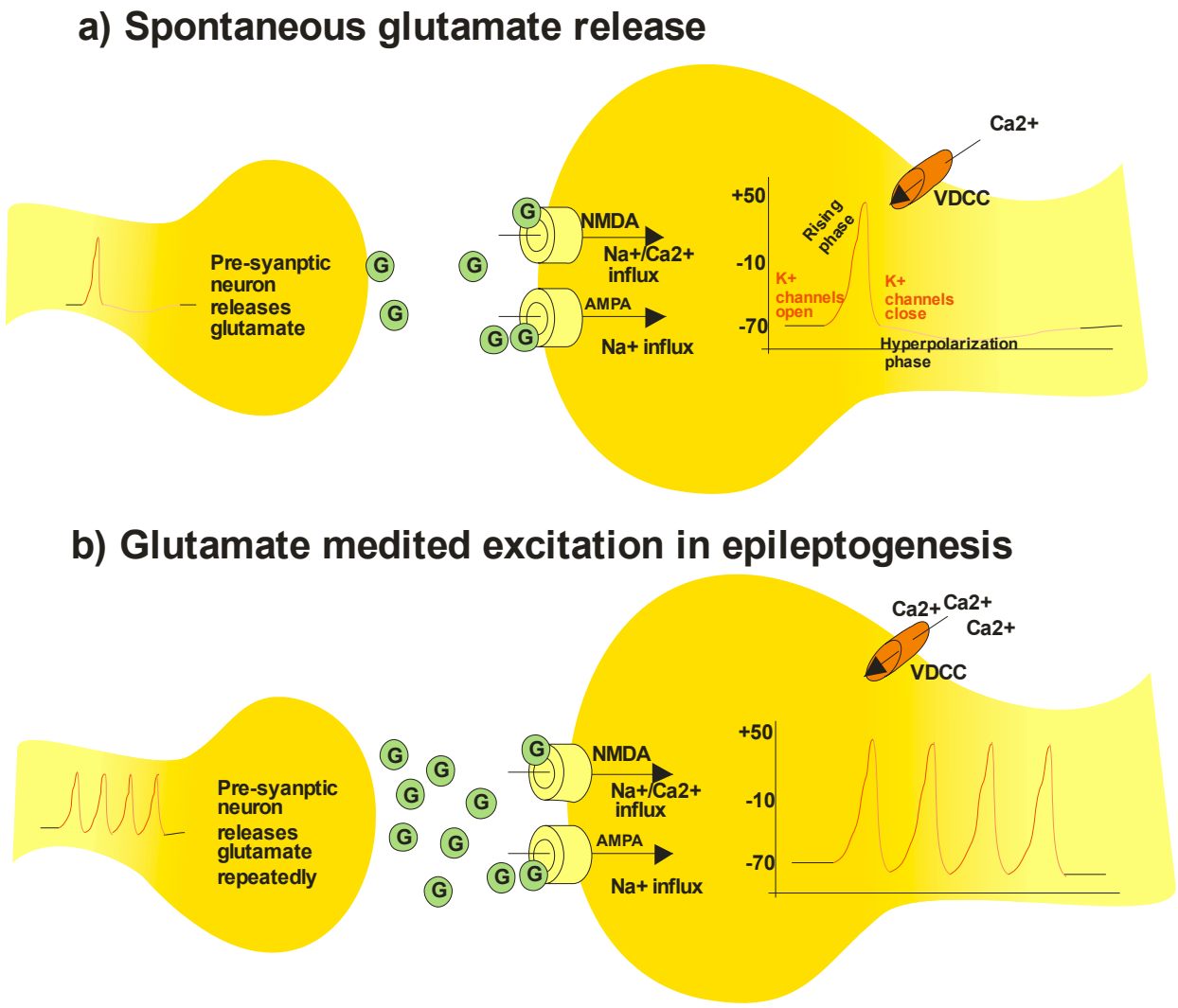


Figure 1. Glutamate mediated excitotoxicity

a) Glutamate is released spontaneously from presynaptic termini. They activate post-synaptic receptors NMDA and AMPA. Activation of AMPA receptors makes them permeable to sodium ions that depolarize the cell. Depolarization opens NMDA receptors that is permeable to calcium in addition to sodium. This calcium along with the calcium from the VDCC leads to further glutamate release. When the cell depolarizes, the potassium channels open and efflux positively charged potassium ions that hyperpolarizes the neuron. This brings the neuron back to the resting membrane potential. b) Increase in glutamate release from the pre-synaptic termini allows repeated neuronal excitation that causes the entry of lethal levels of Ca^{2+} and leads to glutamate excitotoxicity.

The NMDA receptors are made of a NR1 subunit combined to a regulatory subunit NR2A-D [24, 25] . They are a unique subtype of glutamate receptors since they are permeable to Ca^{2+} flow and their activation requires neuronal depolarization [26]. This Ca^{2+} along with the Ca^{2+} from the VDCC leads to an overload that triggers several downstream lethal reactions including nitrosative stress, oxidative stress and mitochondrial dysfunction [16]. AMPA receptors are made of GluR1-4 subunits. They are highly permeable to Na^{+} and K^{+} and are involved in excitotoxicity mediated cell death [27]. Most AMPA receptors in the brain consist of a GluR2 subunit that makes them impermeable to Ca^{2+} [28, 29]. However, GluR2-containing AMPA receptors are decreased during ischemia by down regulation of GluR2 mRNA expression and GluR2 RNA editing, leading to AMPA receptors permeable to Ca^{2+} [30-32]. The KA receptors are made of the GluR5-7 and KA1-2 subunits. Like AMPA receptors, the KA receptors mediate slow neuronal injury [33]. In addition to ionophore-linked receptors, glutamate also activates metabotropic glutamate receptors (mGluRs), a large family of G-protein coupled receptors that modulate excitatory synaptic transmission through several signal transduction mechanisms [34, 35]. Recent advances in the molecular biology, physiology and pharmacology of these receptors revealed their potential role in a variety of central nervous system disorders such as epilepsy, pain, ischemia, and neurodegenerative diseases [36-38].

Excitation and generation of action potentials in a neuron also depends on a diverse array of ion-gated channels for Ca^{2+}, Na^{+}, and K^{+}. The opening of the voltage gated K^{+} channels is a major component that causes repolarization of the cell to the resting potential. This property allows the neuron to curb calcium influx and thus decrease extensive glutamate release [39]. Thus, activation of certain type of endogenous ion channels that are able to attenuate neuronal depolarization may produce neuronal protective effects. One such potential candidate is the

Potassium ATP channels (K_{ATP} channels). A reduction of intracellular ATP levels because of energy deficit activates K_{ATP} channels, thereby preventing membrane epolarization. Hence, the activation of K_{ATP} channels would be able to protect neurons from metabolic stress such as hypoxia and epilepsy.

Molecular basis of K_{ATP} channels

K_{ATP} channels are an octameric complex consisting of inwardly rectifying K^+ channels (Kir6.1 and Kir6.2) and regulatory sulfonylurea receptors (SUR1, SUR2A and SUR2B), which are a member of the ATP-binding cassette (ABC) protein family (Figure 2), [40, 41]. The Kir6.1 and Kir6.2 share 70% amino acid identity and the monomers have a pore loop domain flanked by two transmembrane segments (TM1 and TM2). The N and C-terminal domains are cytoplasmic and bind to ATP. A linker that connects TM1 to TM2 makes the channel selectively permeable to K^+ ion flow [40, 42]. Both the pore-forming subunits and the regulatory SUR receptors have ER retention motifs. In case of the Kir6.2 subunits, the ER retention motif is located at the C-terminal between TM1 and NBF-1 domain. When it associates with SUR1 the ER retention motif is masked, leading to expression of functional Kir6.2/SUR1 channels on the cell surface [43, 44].

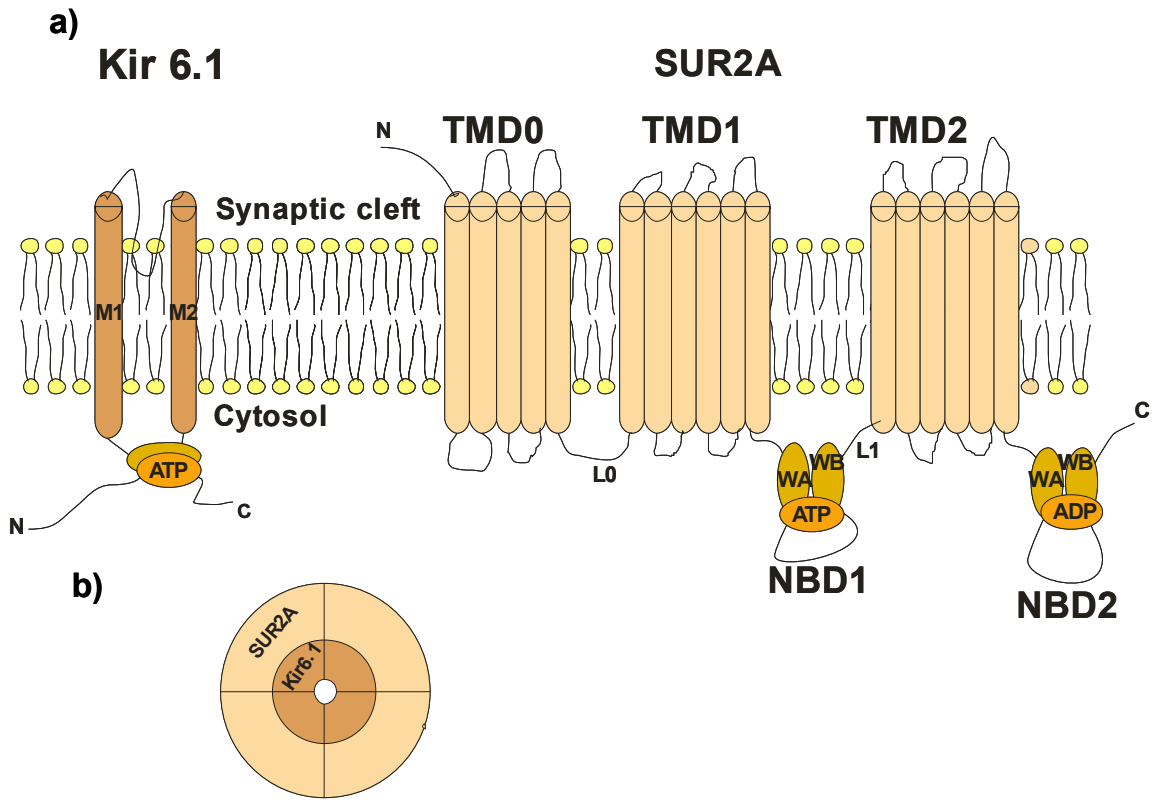


Figure 2. Schematic representation of the structural organization of K_{ATP} channels.

a) K_{ATP} channels have two subunits, Kir and SUR. The Kir is made of two transmembrane domains TM1 and TM2 and a cytoplasmic domain with an inhibitory site for ATP binding. The SUR is composed of three transmembrane domains TMD0, TMD1 and TMD2. They are connected by the linker regions L0 and L1. The Nucleotide Binding domains (NBD1 and NBD2) that bind to ATP are cytoplasmic. They constitute the Walker motifs A and B. b) Heteromeric assembly of K_{ATP} channels where four Kir and four SUR subunits come together to form a functional channel.

There are different types of functional K_{ATP} channels based on the different combination of the Kir and SUR subunits in different cell types. The Kir6.2/SUR1 complex is the β-cell K_{ATP} channels, which controls insulin secretion in response to changes of blood glucose concentration

[45]. The cardiac muscle, K_{ATP} channels consist of the Kir6.2 subunits and SUR2A receptors [46]. The Kir6.1/SUR2B complex forms the vascular K_{ATP} channels and play critical roles in regulation of vascular tonus, especially in the coronary arteries [45]. In contrast to peripheral tissues, neuronal K_{ATP} channels are less understood. To date, the only molecularly characterized K_{ATP} channel components expressed in the brain regions, including the hypothalamus [47], basal forebrain cholinergic neurons [48], and striatum [49], are thought to be the Kir6.2 types. Although pharmacological approaches suggest the presence of SUR1 receptors [50], their functional properties in the brain are yet to be fully characterized. Recently, we used electron microscopy (EM) combined with gene targeting approaches to study neuronal type K_{ATP} channels. Our results demonstrate that, as opposed to the Kir6.2 channels that are most likely to be at peri-synaptic sites, in the hippocampus, functional Kir6.1 channels are predominantly located in the "active zone" of the pre-synaptic terminals that controls glutamate release [51]. Consistent with these data, activation of K_{ATP} channels by channel openers reduces the release of several neurotransmitters, including excitatory glutamate [51], dopamine [49, 52, 53] as well as inhibitory γ-aminobutyric acid (GABA) [49, 54].

K_{ATP} channel function in pancreatic β cells

K_{ATP} channel activity is regulated by nucleotides [55, 56]. Binding of intracellular MgATP to either the SUR or Kir6.2 subunits activates and/or deactivates these channels [40, 57, 58]. Opening of the K_{ATP} channel is also modulated by Phosphatidylinositol bisphosphate (PIP_2) [59, 60] and by mutations of the Kir6.2 gene [55, 61, 62]. The physiological function of the K_{ATP} channel has been extensively characterized in the pancreatic-β cell. These channels are involved in the first phase of insulin secretion. At normal glucose levels, the K_{ATP} channel is active (open). The loss of K^+

maintains the cell at a hyperpolarized state. Increased blood glucose levels and subsequent metabolism increases intracellular ATP levels that inactivate (close) the channel (Figure 3). Subsequent depolarization opens voltage gated Ca^{2+} channels [44]. Increase in intracellular Ca^{2+} concentrations triggers insulin release. As levels of glucose fall, the K_{ATP} channels in the glucose-responsive neurons in the hypothalamus are activated and this stimulates the pancreatic β-cells to secrete glucagon bringing glucose levels to normal [45, 63].

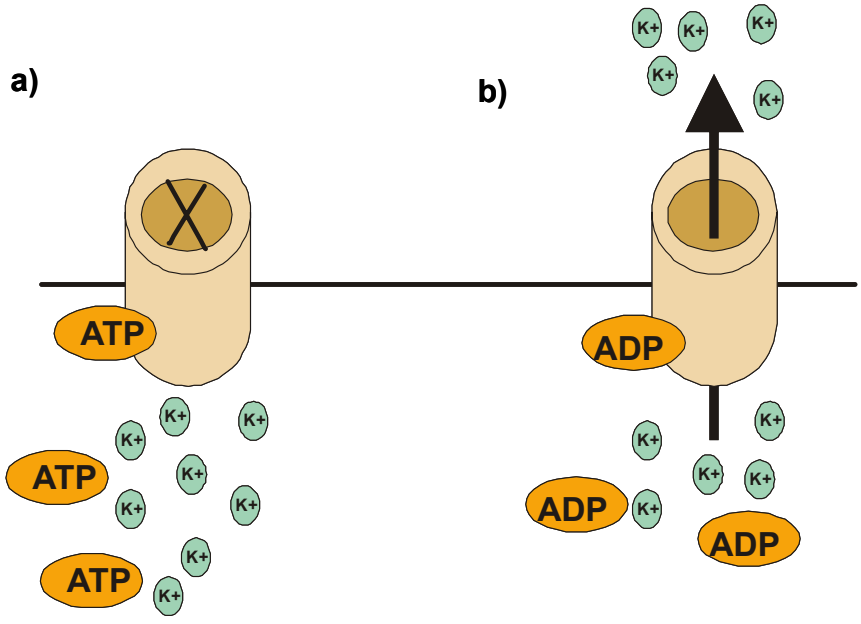


Figure 3. K_{ATP} channel activation is linked to the metabolic status of the cell.

a) In Pancreatic β-cells, increase in glucose, increases ATP levels. This keeps the channels inactive, depolarizing the cell and causing insulin granule release. In the brain, physiological ATP concentrations keep the channel inactive. b) However when the metabolism of the cell is compromised, like during an ischemic or epileptic episode, the levels of intracellular ATP drops and the K_{ATP} channels are activated. Their activation effluxes potassium ions and hyperpolarizes the cell. The loss of membrane potential blocks lethal Ca^{2+} entry.

Accordingly, K_{ATP} channel mutations are associated with many glucose-metabolism related disorders. Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI) or Congenital Hyperinsulinism (CHI) is an inherited disorder due to mutations on the SUR1 or the Kir6.2 genes [64]. Permanent Neonatal Diabetes Mellitus (PNDM) is another disease, which is often associated with mental retardation and epilepsy due to Kir6.2 mutations [65, 66]. Mutations have been reported on the SUR1 subunit as well. Some of these mutations prevent trafficking of the channel to the plasma membrane and others prevent activation of the channel in response to elevated intracellular MgADP [67, 68]. Invariably, all mutations on the K_{ATP} channels decrease insulin secretion and cause improper glucose metabolism [69, 70]. SUR1 knockout mice also have diminished insulin release capabilities and hypoglycemia. Essentially, all these mice exhibit a diabetic phenotype [71].

Several pharmacological compounds that serve as K_{ATP} channel openers or K_{ATP} channel blockers have been useful for characterizing the function of K_{ATP} channels [72]. Diazoxide, cromakalim and pinacidil are major openers of the K_{ATP} channels [58]. Importantly, diazoxide that has been prevalently used to treat insulin release related disorders [73] and is widely used to distinguish the SUR2A-containing K_{ATP} channels from the SUR2B; the SUR2B is sensitive while SUR2A is insensitive to diazoxide [74]. Sulfonylureas including glibenclamide and tolbutamide serve as K_{ATP} channels blockers and are clinically used for the treatment of diabetes [75]. In spite of the availability of these K_{ATP} channel blockers and openers, their lack of specificity is a huge limitation to differentiate between different types of K_{ATP} channels [45].

Mitochondrial K_{ATP} channels

In addition to the K_{ATP} channels on the cell surface, mitochondria have been found to have their own K_{ATP} channels (mitoK_{ATP}) on their inner membrane. Like the cell membrane K_{ATP} channels, they are also inhibited by ATP [76]. Activation of mitoK_{ATP} has shown to exert various protective effects by reducing infarct size during ischemia, blocking cytochrome C release and increasing association of the anti apoptotic Bcl2 protein to the mitochondria [77]. Their major contribution is protection from cell death as shown in ischemic preconditioning during cerebral and cardiac ischemia [78-80]. Diazoxide opens the mitoK_{ATP} channels and 5-hydroxydecanoate blocks it making them widely used compounds to study these channels [72]. The subunit composition of the mitoK_{ATP} is controversial because several groups have attempted to decipher their composition but with conflicting results [81, 82]. This can be attributed to the use of different antibodies raised against different epitopes and their lack of specificity. Research is also hampered by the lack of specific pharmacological tools that would allow for the functional characterization of mitoK_{ATP} and cell membrane K_{ATP} channels [72].

One direct effect of excitatory glutamate toxicity is mitochondrial dysfunction [83, 84]. Increase in cytoplasmic Ca^{2+} through the activation of NMDA (N-methyl-D-aspartate) subtype glutamate receptors results in Ca^{2+} flow into the mitochondria through the Na-Ca uniporter [85]. As the mitochondrial matrix is depolarized the mitochondrial oxidative phosphorylation is impaired, leading to ATP depletion [86]. The swelling of the mitochondria results in membrane rupture and release of pro-apoptotic proteins [87, 88]. In addition to extra cellular Ca^{2+}, mitochondrial stores released Ca^{2+} is also responsible for the intracellular Ca^{2+} overloading and neuronal cell death [89, 90]. Recent studies have shown that activation of mitoK_{ATP} during ischemia reduced Ca^{2+} accumulation in the heart mitochondria [91]. Activation of mitoK_{ATP} by diazoxide has also been

linked to a hyperpolarization in the mitochondria and decreased Ca^{2+} influx [92, 93]. Given that accumulation of Ca^{2+} in the mitochondria also induces reactive oxygen species (ROS)-related neuronal death [94-96], it is reasonable to speculate that neuronal protection can be achieved by activation of mitoK_{ATP} channels during excitotoxic injury.

K_{ATP} channels in glutamate mediated synaptic degeneration

Anoxia-hypoxia depletes cellular oxygen levels and induces anoxic depolarization. This opens several voltage-gated channels leading to a massive overload of Na^+ and Ca^{2+} ions disrupting cellular functions and eventually leading to cell death [97, 98]. Activating K_{ATP} channels in this case will cause anoxic hyperpolarization and dampen the depolarization-induced glutamate excitotoxicity protecting neuronal cells [99]. Several investigators have addressed this issue. For instance, Protein Kinase G (PKG) activates K_{ATP} channels by phosphorylation of serine threonine residues. This contributes to anoxic preconditioning (APC) and was shown to reduce anoxia/reperfusion induced cardiac damage in rat hearts [100]. It has also been shown that zinc protects from ischemic damage of the hippocampus by inhibiting glutamate release from mossy fiber synaptosomes. This was observed when micro molar concentrations of zinc opened K_{ATP} channels during anoxic-hypoglycemic episodes [101].

Recent studies using the null mutant mice lacking the Kir6.2 gene ($Kir6.2^{-/-}$) indicate that the most $Kir6.2^{-/-}$ mice die, whereas all wild-type control mice survive from 15-min occlusion of the middle cerebral artery occlusion (MCAO). Although poor survival of these $Kir6.2^{-/-}$ mice could be due to the surgical procedures but not due to the brain damage [a very small infarction

size (11.67 ± 1.28 mm^3) was observed in these mice] [102], it is generally agreed that expression of K_{ATP} channels protects against neuronal injury from ischemic insults [45, 99].

In addition to ischemic neuronal death, respiratory arrest can induce epileptic activity, which coupled with increased metabolism of the available cerebral oxygen decreases cellular ATP levels and lead to activation of K_{ATP} channels. Increased efflux of K^+ ions mediated by these activated channels causes neuronal hyperpolarization that suppresses the epileptic responses [103, 104]. Indeed the Kir6.2$^{-/-}$ mice exhibit an increased susceptibility to seizures after hypoxia [105]. This can be explained because of the inwardly rectifying nature of the Kir subunit. The subunit helps in hyperpolarizing the membrane following a depolarization and helps control excitability. Conversely, over expression of SUR1 in mice makes them more resistant to generation of seizures [106]. The regulatory nature of the SUR1 subunits probably helps to keep the channel in an active state and thus preventing epileptogenesis.

The Substantia Nigra pars Reticulata (SNr) is a major brain region that controls the propagation of seizures. It also has the highest expression of K_{ATP} channels especially the pancreatic β-cell type (Kir6.2/SUR1) [105]. As the SNr neurons are the central gating system from where seizures propagate, the K_{ATP} channels in the SNr will be the first line of defense against the propagation of seizures caused by ATP depleted conditions such as hypoxia and hypoglycemia. As expected, the firing rate of SNr neurons of Kir6.2$^{-/-}$ mice was 1.8 fold higher compared to the wild type. This effect was reversed by tolbutamide, a sulfonylurea that inhibits K_{ATP} channels [107]. It should be noted here that, the specificity of tolbutamide depends on the availability of Mg^{2+} and K_{ATP} channel subunit composition [75, 108]. Previous studies showed that hypoxic insults activate K_{ATP} channels and in turn suppress epileptic activity in the Substantia

Nigra neurons [105]. Thus, the ability of the K_{ATP} channels to sense the metabolic state of the cell will help reverse the cell damage by reducing membrane excitability.

Kainic acid (KA) is a glutamate analog that serves as an agonist for AMPA/KA receptors [9]. Activation of these receptors leads to a series of intracellular events including Ca^{2+} influx, Reactive Oxygen Species (ROS) production, increased Nitric Oxide Synthase (NOS) levels, membrane depolarization, mitochondrial dysfunction and apoptosis [19]. KA-induced status epilepticus causes delayed and specific neuronal death in the pyramidal neurons of the CA3 and CA1 areas of the hippocampus neurons [9, 109]. This cell death is mainly by the excitation of AMPA type glutamate receptors, which as noted earlier are impervious to Ca^{2+} [28, 29]. Nevertheless, it has been observed that there is a reduction in the GluR2 mRNA expression preceding cell death. Down regulation of GluR2 mRNA facilitates AMPA mediated Ca^{2+} entry into CA3 pyramidal neurons and causing neuronal death [19, 30, 31, 110]. The epileptic activity in the CA3 is also largely generated by the GluR6 receptors [111].

Hypothesis
K_{ATP} channels regulate NMDA receptor function

Glutamate excitotoxicity is a major cause of neuronal cell death during ischemia and seizures. Glutamate is released from pre-synaptic terminals and activates postsynaptic glutamate receptors like NMDA, AMPA and KA [20, 21]. Over-stimulation of NMDA receptors due to glutamate accumulation is lethal as these receptors are permeable to toxic Ca^{2+} ions [112]. Hence, mechanisms that counteract this phenomenon play an essential part in neuronal protection. The ability of the K_{ATP} channels to activate and hyperpolarize the cell in response to a drop in ATP levels places them at a unique position to sense and modulate the activation of glutamate receptors (particularly, NMDA receptors, a principle subtype of glutamate receptors, since they are activated only if membrane potentials are depolarized). Accordingly, activation of K_{ATP} channels will induce neuronal hyperpolarization, prevent NMDA receptor channel opening, and hence protect from hypoxic/ischemic insults and epileptogenesis (Figure 4).

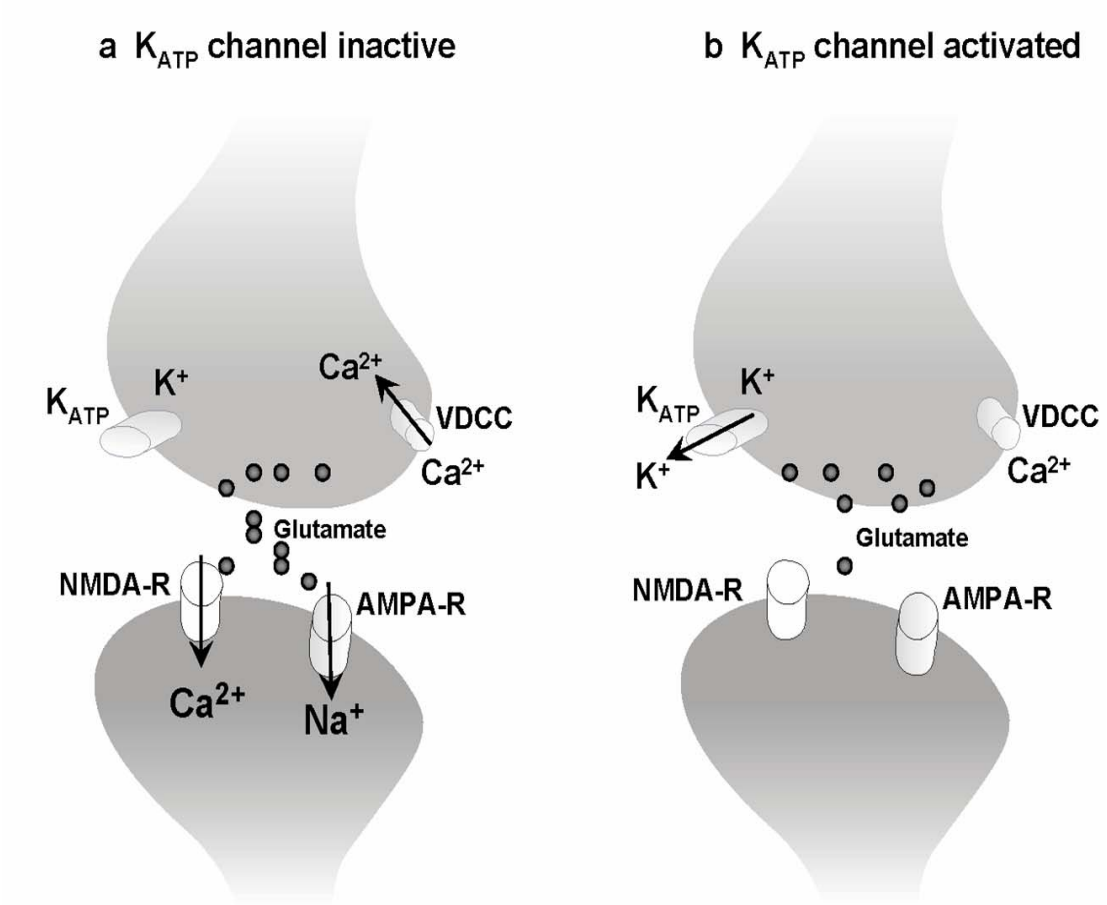


Figure 4. Activation of K_{ATP} channels counteracts glutamate toxicity.

a) K_{ATP} channels are inactive. Hypoxic/ischemia depolarizes neuronal terminals and leads Ca^{2+} influx through voltage-gated Ca^{2+} channels (VDCC). Ca^{2+} entry triggers glutamate release that activates postsynaptic glutamate receptors including NMDA and AMPA receptors. Over-stimulation of NMDA and AMPA receptors leads to Ca^{2+} overloading in the post-synaptic neurons, resulting in neuronal death. b) K_{ATP} channels are activated. Hypoxic/ischemia also activates K_{ATP} channels and in turn hyperpolarizes neuronal terminals. Neuronal hyper-polarization inactivates VDCC and in turn terminates Ca^{2+}-dependent glutamate release, thereby protecting against ischemic insults.

Materials and Methods

Neuron culture

Cortical neurons were cultured from P1 pups. The brains were dissected onto HBSS. The meninges were removed and the cortices were extracted. The tissue was digested with trypsin at 37°C for 15 minutes. The enzyme was inactivated with horse serum and spun at 1000rpm, 5 minutes at 22°C. The pellet was triturated 10 times with a fire polished Pasture pipette. The cell suspension was spun at 1000rpm, 5 minutes at 22°C to pellet the neurons. The cells were resuspended in Neurobasal medium supplemented with B27, 0.5mM Glutamax, PSN, 25μM Glutamate and plated at a concentration of 1 X 10^5 per well. The neurons were then maintained in feeding medium (Neurobasal medium supplemented with B27, 0.5mM Glutamax and PSN) until treatment at 37°C, 5% CO_2.

Stable Kir6.1 gene silencing and virus infection

Kir6.1 siRNA (KsiRNA) sequence (forward: 5′-GGUGAAUGGCCAGGUUUUUtt-3′; reverse: 5′-AAAAACCUGGCCAUUCACCtc-3′) was provided by Ambion, Inc. (Austin, TX) and synthesized as two cDNA oligonucleotides, which were cloned in *p*Silencer-U6 through *BbsI*/*BstB* sites, as described before [31, 32, 113]. The U6-KsiRNA cassette was re-cloned into rAVE^{TM} construct containing eGFP through *ApaI*/*KpnI* (GenDetect, New Zealand), creating a vector rAVE-U6/KsiRNA-CAP/eGFP. A control vector was used to express a scrambled KsiRNA (rAVE-U6/SKsiRNA-CAP/eGFP). The rAVE plasmids were co-transfected with the

AAV helper 2 into HEK293 cells to generate the rAAV2 virus particles. The high titer infectious virus particles were purified and calculated by GenDetect Inc. (New Zealand). The infectious particles were then perfused (2 µl at 0.2 µl/min) into each side of the hippocampus (2.1 mm posterior to bregma; 1.9 mm lateral to the midline; 2.6 mm below dura) of mice, as previously described [31, 32, 113]. 12 days after injection, the mice were subjected to experimentation.

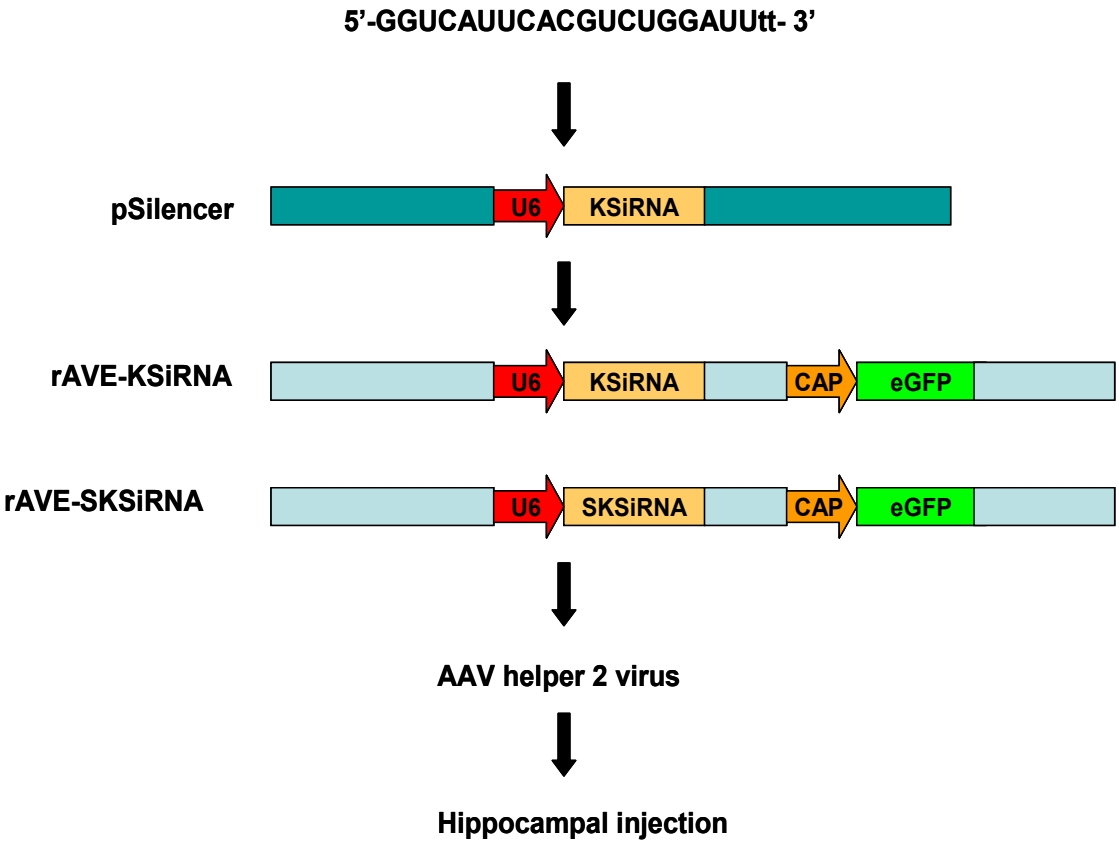


Figure 5. Flowchart representing the construction of Kir 6.1 SiRNA in an Adeno virus Associated helper virus.

RT-PCR analysis

RNA was isolated from the hippocampus of mice without or with infection of the virus particles and treated with the DNA-free™ kit (Ambion), as described before [31]. Sample aliquots containing 25 µg RNA were incubated for 30 min at 37°C with 2 IU DNase I in DNase reaction buffer [10 mM Tris–Cl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$] in a total volume of 50 µl. The reaction was stopped by addition of 5 µl resuspended DNase Inactivation Reagent and incubation at room temperature for 2 min. This reagent, as well as the DNase I and divalent cations, were removed by centrifugation at 10000x*g* for 1 min. DNase I-treated RNA samples were reverse-transcribed in a 20 µl reaction volume containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 ng oligo$(dT)_{15}$ primer and 200 IU M-MLV reverse transcriptase (Promega, Madison, WI) for 1 hr at 42°C. Control samples, where no reverse transcriptase was added, were included in all experiments to show that all products were RNA-derived and not the result of genomic DNA contamination. PCR amplification was carried out with 5 µl cDNA product in a 50 µl reaction volume containing 20 pmol of each specific oligonucleotide primers: Kir6.1 nucleotides 277-615 (accession no. NM-008428) forward: 5'-AAAGGAAGATGTTGGCCAGGAA-3', reverse: 5'-CCATGGTGCCTTTCTCCATGTA-3', 50 nmol/l dNTP, 1.25 IU Taq DNA Polymerase (5 IU/µl) (Roche Diagnostics, Mannheim, Germany) in 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3). After an initial pre-heat at 95°C, PCR amplification was carried out for 22 to 45 cycles of denaturation at 95°C (20 s), annealing at 52°C (45 s), and extension at 72°C (1 min), followed by a final extension at 72°C for 10 min. The number of cycles used was limited to ensure product amplification remained in the log-linear range. ß-actin was adopted as a control. PCR products were separated by

electrophoresis on a 1.5% agarose gel and visualized after ethidium bromide staining over UV light.

Electron microscopy

The post-embedding immuno-gold method has been described [114, 115]. In this study, sections from the CA1 stratum radiatum of the hippocampus of 3 male Sprague−Dawley rats (37 days old). Rats were perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.12 M phosphate buffer. Parasagittal sections (250 μm) of the hippocampus were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica EM CPC. Frozen sections were immersed in 1.5% uranyl acetate in methanol at −90°C in a Leica AFS freeze−substitution instrument, infiltrated with Lowicryl HM-20 resin at −45°C, and polymerized with ultraviolet light. Thin sections of the hippocampus were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris−buffered saline/0.1% Triton X−100 (TBST), followed by 10% normal serum (NS) in TBST. One set of the experiments were done using a 10-day old rat, anti-Kir6.1 (1:300) and anti-Kir6.2 (1:300) antibodies were provided by Dr Andrew Tinker (University College London, London, UK). Another set of the experiments were done on three adult rats, using anti-rabbit Kir6.1 antibody (1:80) purchased from Sigma (P0874) and anti-goat Kir6.2 antibody (10 μg/ml) purchased from Santa Cruz (SC-11226). The antibodies were prepared in 1% NS/TBST and 10nm immunogold was prepared in 1% NS/TBST plus 0.5% polyethylene glycol, and finally the sections were stained with uranyl acetate and lead citrate. The anti-Kir6.1 antibody was raised in rabbits against a peptide corresponding amino acids 399-RRNNSSLMVPKVQFMTPEGNQC-420) and anti-Kir6.2 was raised in goats against Kir6.2 peptide corresponding amino acid 357-

372. For quantification of random areas of the CA1 stratum radiatum from the 3 P37 rats, all spine synapses with gold labeling within 20 nm of the cell membrane of the presynaptic terminal and the postsynaptic spine were photographed and counted. The membrane was divided into 6 regions including the synaptic (=active zone), perisynaptic (=100 nm on either side of the active zone), and extrasynaptic membrane of both the presynaptic terminal and the postsynaptic spine membrane. A total of 56 synapses for Kir6.1 and 59 synapses for Kir6.2 were counted from the 3 animals. Statistical analysis used a two-tailed t-test.

Kainate administration

Adult male mice (90 ± 5 days old of age, 28 ± 2 mg body weight) were intraperitoneally injected with kainic acid (30 mg/kg in PBS, A. G. Scientific Inc., San Diego, CA). Mice were monitored continuously for 3 hrs. The severity of seizures was rated by the arbitrary scale: 1: staring and immobility/wet dog shake; 2: hyperactivity, repetitive movements, rearing and falling; 3: low seizures (intense shivering); 4: severe tonic/clonic convulsion; 5: death. The averaged points for seizure severity in a given group were expressed as the seizure index.

Immunoprecipitation

Synaptosomes were generated as described before [31, 32, 113]. Briefly, the hippocampal homogenate in 0.32 M sucrose was centrifuged for 10 min at 1,400g to yield a pellet (P1) and a supernatant (S1). S1 was further centrifuged for 10 min at 13,800g, yielding a crude synaptosomal pellet (P2) and a supernatant (S2). P2 was resuspended in 0.32 M sucrose containing 1 mM $NaHCO_3$ and layered on top of a discontinuous sucrose gradient (0.8, 1.0 and

1.2 M). After centrifugation for 2 h at 82,500g, the synaptosomes were recovered as a band, resuspended in 0.32 M sucrose and 1 mM NaHCO3 plus protease inhibitors, pelleted and resuspended in HEPES buffer containing protease inhibitors.

Synaptosomes were incubated with 1% Triton X-100 for 20 min on ice and centrifuged at 14,000g for 15 mins to obtain the supernatant. Protein concentration in the extracts was determined by Lowry assay (Bio-Rad). The extracts (~500μg protein) were incubated with polyclonal rabbit anti-SUR1 (2 μg) overnight at 4°C, followed by the addition of 40 μl of Protein G-Sepharose (Sigma) for 3 hr at 4°C. Immunoprecipitates were washed four times with PBS buffer, denatured with SDS sample buffer, separated by SDS-PAGE and blotted with anti-Kir6.1 (Santa Cruz, 1:100), anti-SUR1, or anti-syntaxin-1A (Santa Cruz, 1:400) antibodies. Anti-SUR1 antibody was raised in a rabbit against the synthetic peptide CKRGAILEFDKPEKL (Sigma). The antibody was purified by using an immunoaffinity column.

Electrophysiology

Hippocampal slices (250 μm) were prepared from mice, as described before [31, 32, 113]. Slices in the recording chamber were continuously superfused with artificial cerebrospinal fluid (ACSF, 2 ml/min) saturated with 95% O_2/5% CO_2 at 30°C ± 1°C. The composition of ACSF was 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM dextrose. For whole-cell patch-clamp recordings (tight seal >15-25 GΩ) from CA3 pyramidal cells, hippocampal slices were visualized with IR-DIC optics with an Axioskop 2FS equipped with Hamamatsu C2400-07E optics. The spontaneous EPSCs were recorded at a holding potential of -70 mV. The intracellular solution contained 142.5 mM Cs-gluconate, 7.5

mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM Mg-ATP, 0.3 mM guanosine triphosphate (pH 7.4; 296 mOsm). The currents were filtered at 5 kHz with a low-pass filter. Data were digitized at a frequency of 10 kHz and stored online with the pclamp11 system (Axon Instruments, Inc., Foster, CA). The input resistance and series resistance in postsynaptic pyramidal cells were monitored with prevoltage steps (−2 mV; 100 ms) at 5 min intervals throughout the period of the experiment. Series resistance ranged from 11 to 17 MΩ. Input resistance was 346 ± 18 MΩ.

Treatment of cortical neurons with glutamate receptor antagonists

Primary cortical neurons on DIV14 were washed twice with Neurobasal medium and treated with the following

i) vehicle

ii) 400μM Kainic acid + 10μM MK-801 + 10μM CNQX

iii) 400μM Kainic acid

iv) 500μM glutamate + 10μM MK-801 and

v) 500μM glutamate

in Neurobasal for one hour. At the end of treatment the cells were washed 4X with complete Neurobasal medium and let to recover in feeding medium (Neurobasal medium supplemented with B27, 0.5mM Glutamax and PSN) for 5 hrs at 37°C, 5% CO_2. The medium was recovered to measure LDH release and the cells were stained with propidium iodide.

LDH release assay

The LDH release assay was carried out according to the manufacturer's protocol (Promega cytotox 96 Non-radioactive cytotoxicity assay kit). Briefly, 50µl of the medium was mixed with 50µl of freshly reconstituted substrate in a 96 well plate. The plate was incubated in the dark for 30 minutes at 22°C. The reaction was terminated with 50µl of stop solution and the absorbance was read at 490nm. The average LDH release was calculated from 4 independent experiments. The value was expressed as a percentage of LDH release normalized over glutamate mediated cell death (treatment v) using the formula

% Cell death = [(absorbance treatment – absorbance control) X 100] / [absorbance glutamate].

Propidium iodide staining

The cells were washed three times with Hanks Balanced Salt solution (HBSS) to remove the culture medium. They were stained with Propidium iodide (PI) (3µM) in HBSS for 20 minutes at 37°C, 5% CO_2 followed by 4X wash with HBSS. The stained cells were fixed with 4% para formaldehyde in PBS pH 7.4 at 22°C for one hour. The cover slips were then mounted on glass slides using DPX Fluor mounting medium. The pictures were taken with a Carl Zeiss Apotome microscope. The number of PI positive cells were counted manually and expressed as a percentage of total cells in the field.

Results

The Kir6.1 and Kir6.2 proteins are common at excitatory synapses

We first examined the expression of K_{ATP} channels in the hippocampus by immuno-gold labeling of the Kir6.1 (Fig. 6a) and Kir6.2 (Fig. 6b) subunits. Overall, the labeling shows that both Kir6.1 and Kir6.2 proteins are found on synaptic membranes of terminals and spines as well as in vesicular structures within the synaptic cytoplasm.

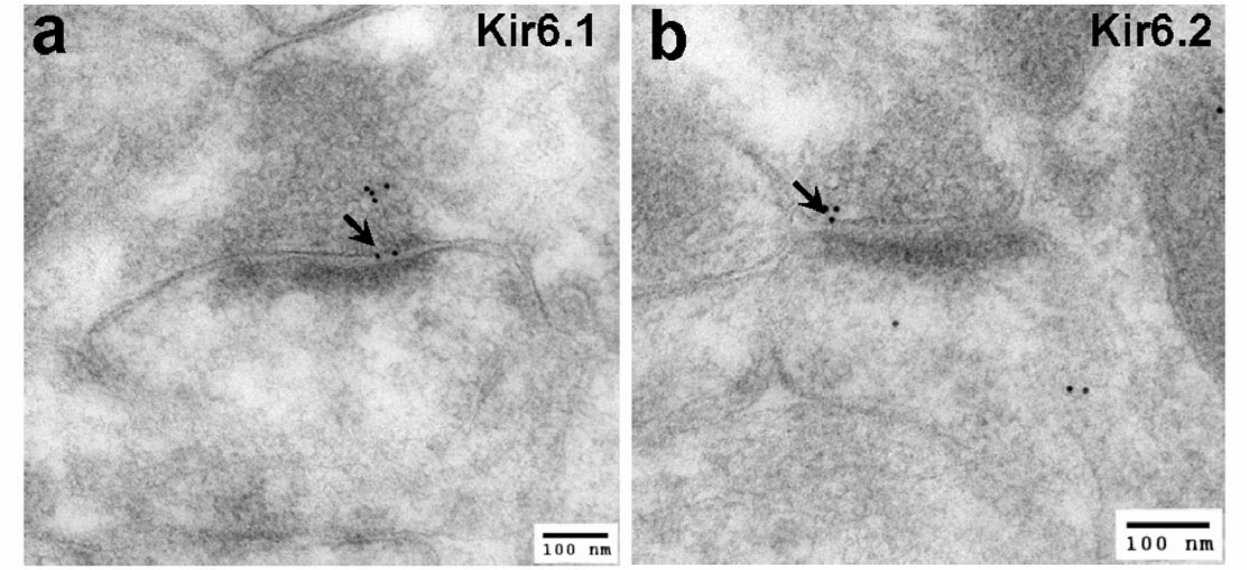


Figure 6. Expression patterns of K_{ATP} channels in the hippocampus.

Representative micrographs showing immunogold labeling of the Kir6.1 (a) and the Kir6.2 (b) Proteins associated with synapses in the adult rat hippocampus. Arrows indicate the labeling in the pre-synaptic (a) and pre-perisynaptic (b) membrane.

By comparing the labeling patterns between Kir6.1 and Kir6.2 proteins, we found that the Kir6.1 subunits were located predominantly in the presynaptic membrane (active zone), whereas Kir6.2 subunit was most likely to be located in the perisynaptic area of the terminals (Fig. 7a and 7b). These findings indicate that Kir6.1 proteins in the presynaptic terminal are concentrated within the synaptic active zone.

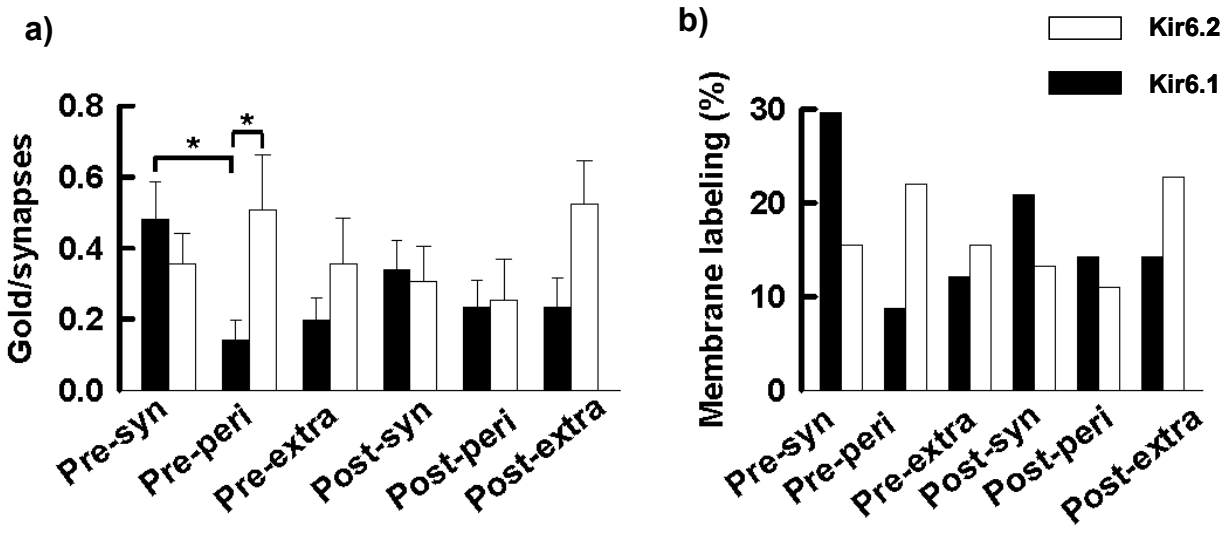


Figure 7. Bar graphs showing presence of Kir6.1 at the pre-synaptic terminal.

a) Bar graph summarizes the gold/synapse for the Kir6.1 (filled bars) and the Kir6.2 (open bars) labeling in pre-synaptic (pre-syn), pre-perisynaptic (pre-peri), pre-extrasynaptic (pre-extra), post-synaptic (post-syn), post-perisynaptic (post-peri) and post-extrasynaptic (post-extra) membrane. Data are mean ± SEM (*$p < 0.01$). The left asterisk indicates the difference between Kir6.1 pre-synaptic and pre-peri-synaptic ($p < 0.01$); and the right asterisk indicates the difference between Kir6.2 and Kir6.1 pre-perisynaptic ($p < 0.05$). b) Bar graph shows a percentage of total membrane gold for the Kir6.1 (filled bars) and the Kir6.2 (open bars). Data in (a) and (b) are mean from three 37 days old mice.

Generation of the Kir6.1 stable gene silencing in the hippocampus of adult mice

We next determined the potential functions of the synaptic K_{ATP} channels in the hippocampus. We generated stable Kir6.1 gene silencing using small interfering RNA (siRNA) that specifically targets to the Kir6.1 gene (KsiRNA). The KsiRNA was expressed in the hippocampus of adult mice using the rAAV2-KsiRNA-eGFP vectors (Fig. 8)

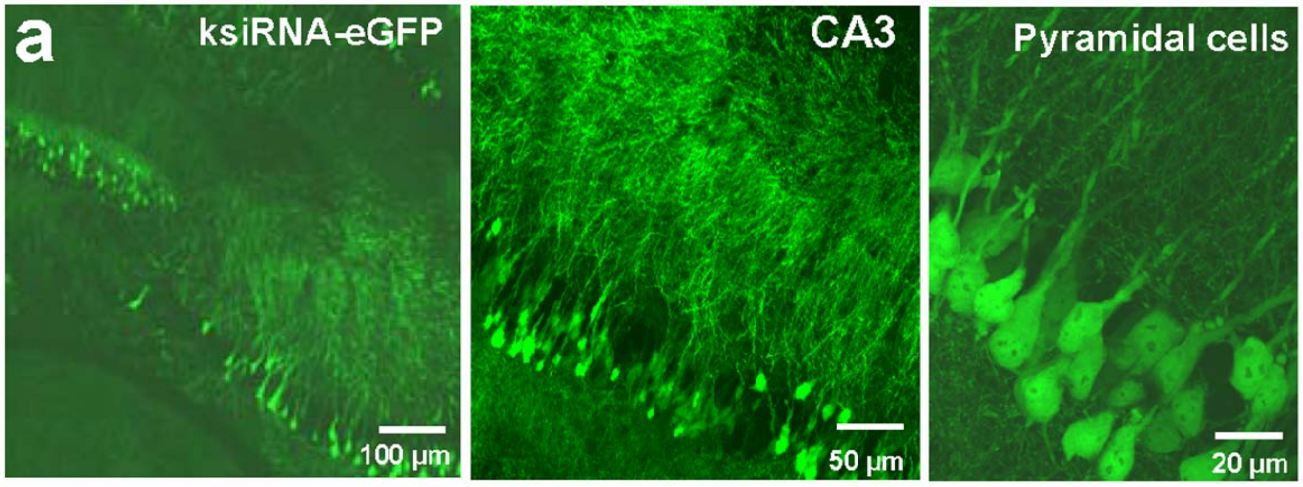


Figure 8. Inhibition of the Kir6.1 gene expression in the hippocampus

The representative images of the hippocampus (left) expressing the KsiRNA and eGFP were taken 12 days after injection of the infectious rAAV2-ksiRNA-eGFP virus particles. Higher magnifications show the CA3 area of the hippocampus (middle) and pyramidal neurons (right).

Expression of a scrambled KsiRNA (SKsiRNA, rAAV2-SKsiRNA-eGFP vector) was used as control. The Fluorescence images show that neurons expressing a reporter gene (eGFP) were present throughout the hippocampus 12 days after infection with the rAAV2 virus particles. A reduction of the Kir6.1 mRNA levels was detectable 6 days after infection with the rAAV2-KsiRNA-eGFP, and the maximum inhibition of the Kir6.1 mRNA (Fig. 9a and b, 76.9 ± 5.3%, n

= 6 assay/3 mice) was observed 6 days later (12 days after virus infection), demonstrating a stable Kir6.1 gene silencing in the hippocampus of adult mice *in vivo*

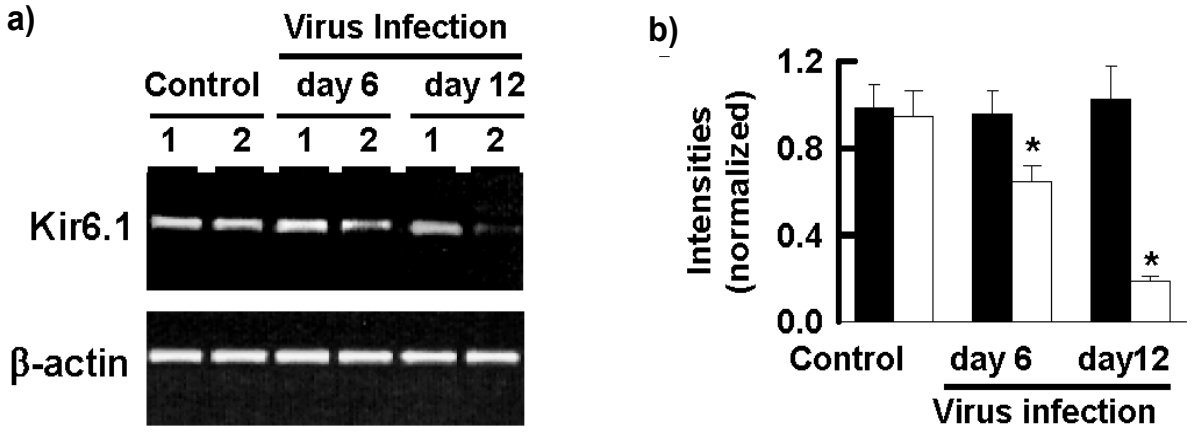


Figure 9. KSiRNA effectively downregulates Kir6.1 mRNA levels

a) RT-PCR analysis shows the Kir6.1 mRNA in the hippocampus of mice 6 days or 12 days after without (control) or with infection of the rAAV2-sksiRNA-eGFP (lane 1) or the rAAV2-ksiRNA-eGFP (lane 2) virus particles. b) Bar graph shows the Kir6.2 mRNA levels that were normalized to β-actin in their respective conditions. Data are mean ± SEM (* $p < 0.01$).

Silencing Kir6.1 gene increases the susceptibility of mice to KA-induced seizures

Subsequently, we analyzed the susceptibility of adult mice with Kir6.1 gene silencing to kainic acid (KA)-induced seizures, which are widely considered to be an animal model of human temporal lobe epilepsy and are associated with hyper-excitation in some subsets of neurons in the hippocampus [9]. Mice were injected with KA (30 mg/kg, i.p.) 12 days after infection with the rAAV2-KsiRNA-eGFP (KsiRNA mice) or the control rAAV2-SKsiRNA-eGFP (SKsiRNA mice) virus particles. The latency and severity of seizures were then diagnosed and expressed as

seizure index. Our data, as summarized in Table 1, show that all the KsiRNA mice suffered severe seizures including hyperactivity, constant rearing and falling. 7 of 10 KsiRNA mice had tonic convulsion and died within 3 hr (seizure index = 4.4 ± 0.31, n = 10). In contrast, the majority of the SKsiRNA mice showed no hyperactive responses and remained alive throughout the course of observation (seizure index = 2.56 ± 0.34, n =10).

Table 1. Seizure severity and latency of onset after administration of kainate in Kir6.1 silenced mice.

Experimental Groups	No. of mice dead (% mortality)	Seizure Index (mean ± SEM)	Latency (min) (mean ± SEM)
SKsiRNA	**2/10 (20%)**	**2.56 ± 0.34**	**30.1 ± 1.1**
KsiRNA	**7/10 (70%)**	**4.40 ± 0.31***	**34.5 ± 3.7**

The SKsiRNA and KsiRNA represent the mice that were intra-hippocampally injected with the rAAV2-SKsiRNA-eGFP or the rAAV2-KsiRNA-eGFP virus particles, respectively. The Kir6.1 silenced mice show increased susceptibility to kainic acid induced epileptogenesis and exhibit higher mortality rates compared to the control, scrambled SiRNA injected mice. *p < 0.01, compared to controls.

To determine the functional partners of the Kir6.1 channels in the hippocampus, we precipitated the Kir6.1 protein complex in synaptosomes. Western blot analysis demonstrated the presence of the SUR1 proteins in the precipitates, indicating that the Kir6.1 subunits form a complex with SUR1 receptors. In addition to SUR1, we also found Syntaxin 1A, one of the SNARE proteins that participate in transmitter release, in the Kir6.1 protein complex (Fig. 10). These results confirm the labeling of functional Kir6.1/SUR1 channels at pre-synaptic terminals.

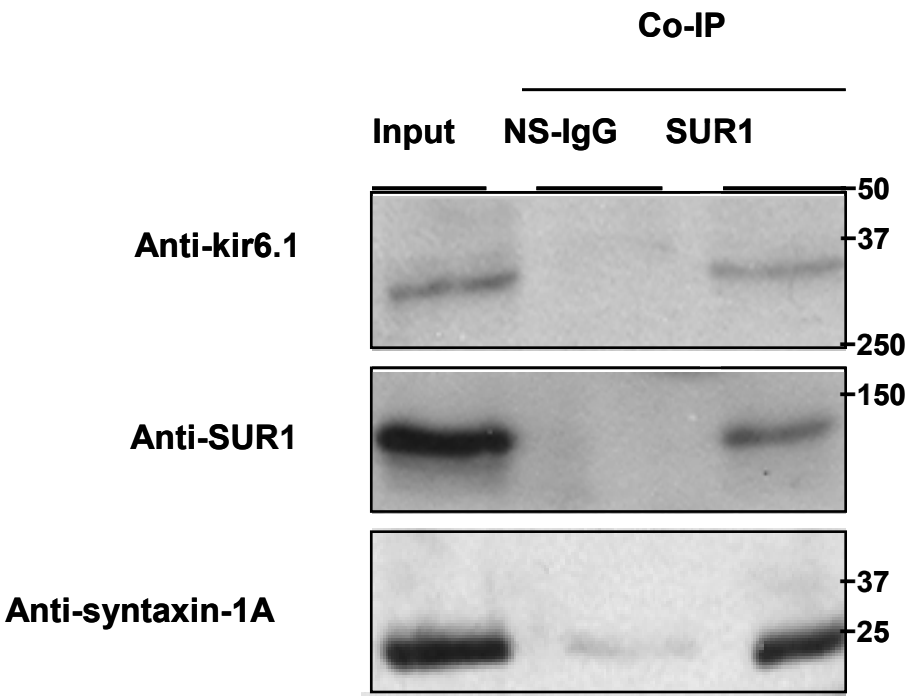


Figure 10. The Kir6.1 is physically associated with SUR1 proteins.

Endogenous Kir6.1 and SUR1 proteins in synaptosomes from adult mouse hippocampus were precipitated with nonspecific IgG or anti-SUR1, and blotted with anti-Kir6.1, anti-SUR1, or anti-syntaxin-1A, as indicated. In the lane marked input, 50 μg of proteins without immunoprecipitation was loaded. Similar results were observed in four independent experiments.

The null mutant mice lacking the SUR1 gene are vulnerable to KA-induced seizures

Accordingly, we examined the seizure vulnerability of the null mutant mice lacking the SUR1 gene ($SUR1^{-/-}$). We found that 8 of 11 $SUR1^{-/-}$ mice revealed tonic and clonic convulsion and died within 3 hr after seizure induction (seizure index = 4.73 ± 0.14), whereas most wild-type control ($SUR1^{+/+}$) mice survived (seizure index = 1.73± 0.36, n = 11, Table 2) and did not exhibit severe seizures. In addition to Kir6.1, the Kir6.2 subunits also form function channels with SUR1 receptors in the pancreatic β-cells [45] and possible in some subsets of neurons in the

brain [47-49, 105]. We found, however, that the mutant mice lacking the Kir6.2 gene (Kir6.2$^{-/-}$, seizure index = 2.56 ± 0.34, n =12, Table 1) did not show a difference in the latency and severity of seizures compared with that of wild-type control (Kir6.2$^{+/+}$) mice (seizure index = 1.73 ± 0.36, $p > 0.01$, n = 11, Table 2). Together, these results demonstrate a crucial inhibitory role of the Kir6.1/SUR1, but not of the Kir6.2 channels, in generation of hippocampus-dependent epileptic form of seizures.

Table 2. Seizure severity and latency of onset after administration of kainate in SUR1$^{-/-}$ mice.

Experimental Groups	No. of mice dead (% mortality)	Seizure Index (mean ± SEM)	Latency (min) (mean ± SEM)
Controls	**1/11 (9.1%)**	**1.73 ± 0.36**	**[a]N/A**
SUR1$^{-/-}$	**8/11 (73%)**	**4.73 ± 0.14***	**32.3 ± 2.0**
Kir6.2$^{-/-}$	**2/12 (16%)**	**2.50 ± 0.34**	**38.1 ± 1.7**

Controls are wild-type C57BL/6 mice. The SUR1$^{-/-}$ mice show increased susceptibility to kainic acid induced epileptogenesis and exhibit higher mortality rates compared to wild type controls as well as the Kir6.2$^{-/-}$ mice. [a]Data are not available since only one mouse in this group suffered seizures within 3 hrs after treatment of KA. *$p < 0.01$, compared to controls.

Enhanced glutamate release in neurons lacking the Kir6.1 channels

Generation of epileptic form of seizures largely results from release of excitatory glutamate transmitter at CA3 synapses in the hippocampus [9]. Given that the Kir6.1 proteins are found to be located at pre-synaptic terminals, we hypothesized that the neurons lacking the Kir6.1 channels may have enhanced release of glutamate transmitter for the induction of seizures. To test this hypothesis directly, we recorded the spontaneous excitatory postsynaptic currents (sEPSCs) from CA3 pyramidal neurons in the hippocampus of the KsiRNA (Fig. 11a) and the KSsiRNA mice (Fig 11b). The frequency of the sEPSCs onsets during KA application in the KsiRNA mice (2.12 ± 0.23 Hz, mean ± SEM, n = 12/recordings/4 mice Fig. 11c) were increased, compared to the KSsiRNA mice (1.23 ± 0.21 Hz, mean ± SEM, n = 12/recordings/4 mice). The mean amplitudes were statistically identical between groups (Fig. 11d).

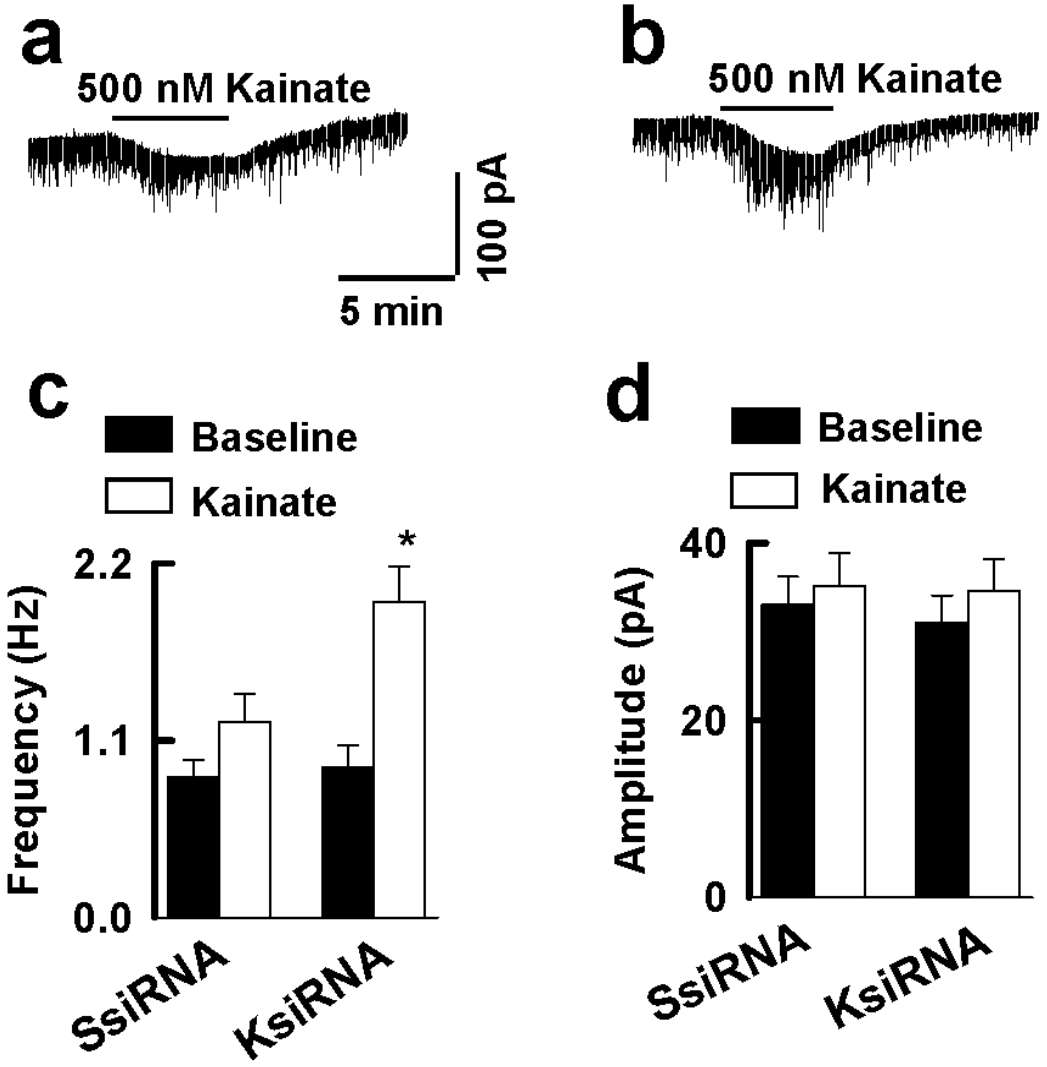


Figure 11. Inhibition of Kir6.1 gene expression enhances KA-induced glutamate release at CA3 synapses.

(a-d) The traces are representatives of the spontaneous $EPSCs_{AMPA}$ recorded at holding potential of -70 mV from CA3 pyramidal neurons in the hippocampus expressing scrambled siRNA (SsiRNA, a) or the Kir6.1 siRNA (KsiRNA, b). Horizontal bar above the traces indicates kainate application. Bar graphs summarize the frequency (c) of the $EPSCs_{AMPA}$ onset and the mean amplitudes of the $EPSCs_{AMPA}$ (d). Data are mean ± SEM (n = 12 recordings/4 mice/group, $*P < 0.01$).

Enhanced glutamate release in neurons lacking the SUR1 subunits

Following our data the SUR1 seems to be the regulatory component of the Kir6.1 containing K_{ATP} channels in the hippocampus. Therefore, we hypothesized that their absence might also increase glutamate release from pre synaptic terminals. Therefore, we recorded the sEPSCs in CA3 pyramidal neurons from the $SUR1^{-/-}$ and the $Kir6.2^{-/-}$ mice (Fig. 12a). In this line of the experiments, the sEPSCs variability was estimated. The distribution of the sEPSCs at the baseline had clearly distinguishable peaks, and was fitted by the sum of multiple Gaussian functions (Fig. 12a). The frequency of sEPSCs was increased following application of KA, whereas the mean amplitude was unchanged (Fig. 12c). By comparison of the recordings between CA3 pyramidal neurons from the $SUR1^{-/-}$ and wild-type control mice, our results indicate that genetic deletion of SUR1/Kir6.1 channels increases glutamate transmitter release at CA3 synapses.

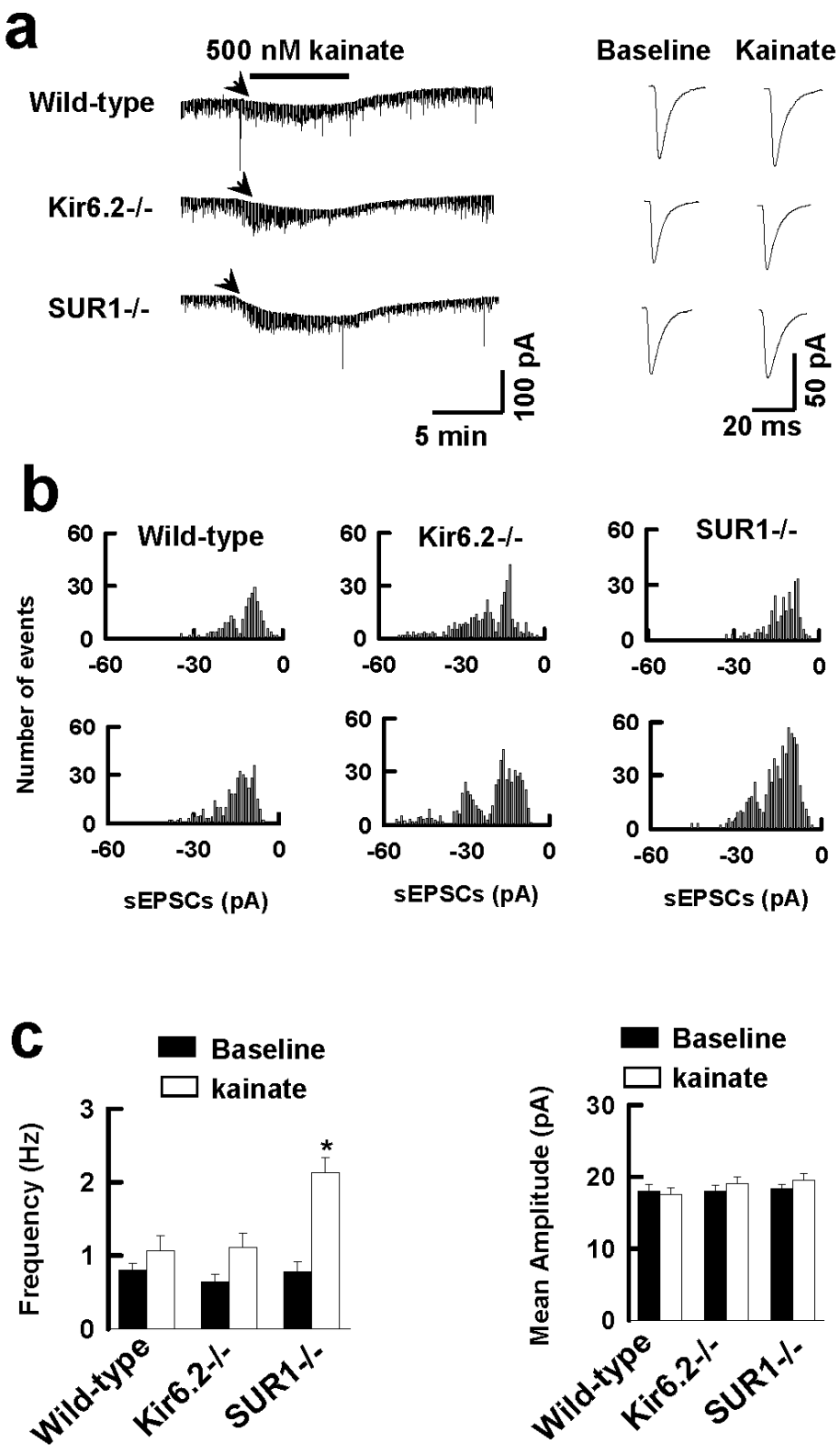

a
500 nM kainate
Baseline
Kainate
Wild-type
Kir6.2-/-
SUR1-/-
100 pA
5 min
50 pA
20 ms
b
Wild-type
Kir6.2-/-
SUR1-/-
Number of events
60
30
0
-60
-30
0
sEPSCs (pA)
c
Baseline
kainate
Frequency (Hz)
3
2
1
0
*
Wild-type
Kir6.2-/-
SUR1-/-
Mean Amplitude (pA)
30
20
10
0

Figure 12. Kainate increases glutamate release at CA3 synapses in the hippocampus.

(a) Representative traces are from whole-cell patch clamp recordings of spontaneous EPSCs at holding potentials of -70 mV from CA3 pyramidal neurons in the hippocampus from wild-type controls (n = 14 cells/10 mice), or the Kir6.2$^{-/-}$ (n = 12 cells/7 mice) or the SUR1$^{-/-}$ (n = 15 cells/7 mice) mice. Averaged spontaneous EPSCs before and during KA are shown in the right. Horizontal bar above the traces indicates kainate application. Note that kainate produced depolarizing currents (arrows) in the CA3 pyramidal neurons, but the mean amplitudes of the currents were no difference among groups. (b) Amplitude distribution histograms for the spontaneous EPSCs before (top) and during (bottom) KA are plotted with bin sizes of 4 pA. (c) The frequency but not the mean amplitude is increased at CA3 synapses in the hippocampus from the SUR1$^{-/-}$ mice. Data are mean ± SEM (* $p < 0.01$).

Kainic acid induced cell death is higher in SUR1$^{-/-}$ mice

Our observations along with others have shown that although C57BL/6 mice undergo epileptic seizures on treatment with kainic acid, they do not show any neuronal cell death *in vivo*. To test this effect *in vitro* we treated primary cortical neurons from wild type controls, SUR1$^{-/-}$ and Kir6.2$^{-/-}$ mice with kainic acid (Figure 13). The cell death in these neurons was measured in terms of the LDH released. The K_{ATP} channel deficient neurons treated with kainic acid showed increased susceptibility to cell death as compared to wild type controls. This susceptibility was completely abolished in all genotypes when the glutamate receptor activation was blocked with specific blockers MK-801 (NMDA) and CNQX (AMPA). This shows that the kainic acid mediated excitotoxicity is mediated by glutamate activating postsynaptic glutamate receptors. It also shows that this excitotoxicity can be effectively counteracted by functional K_{ATP} channels.

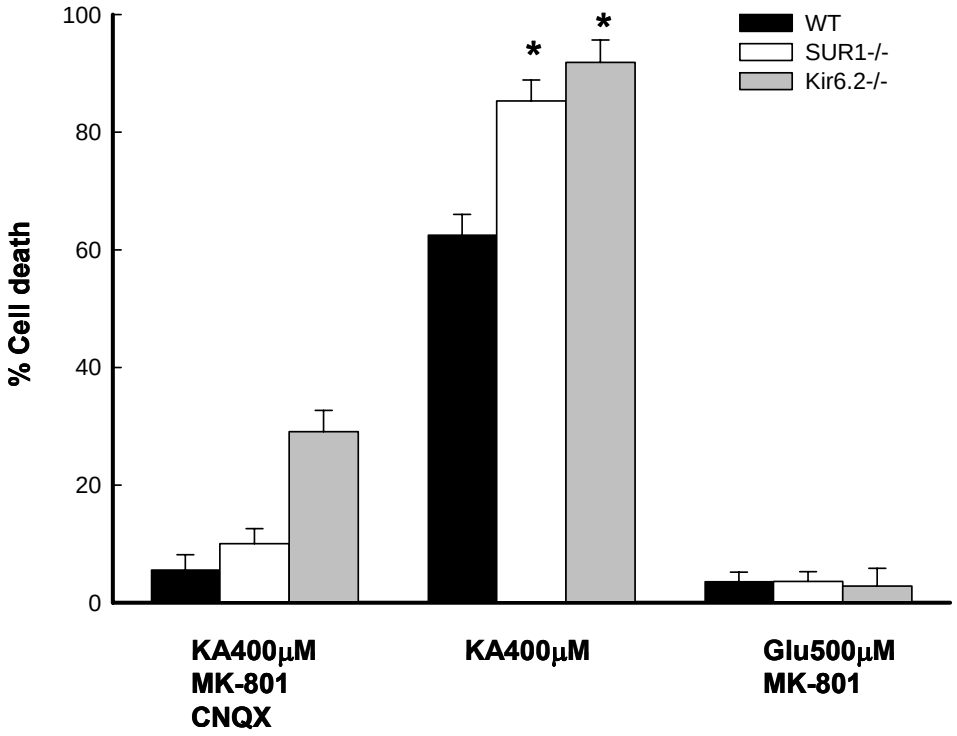
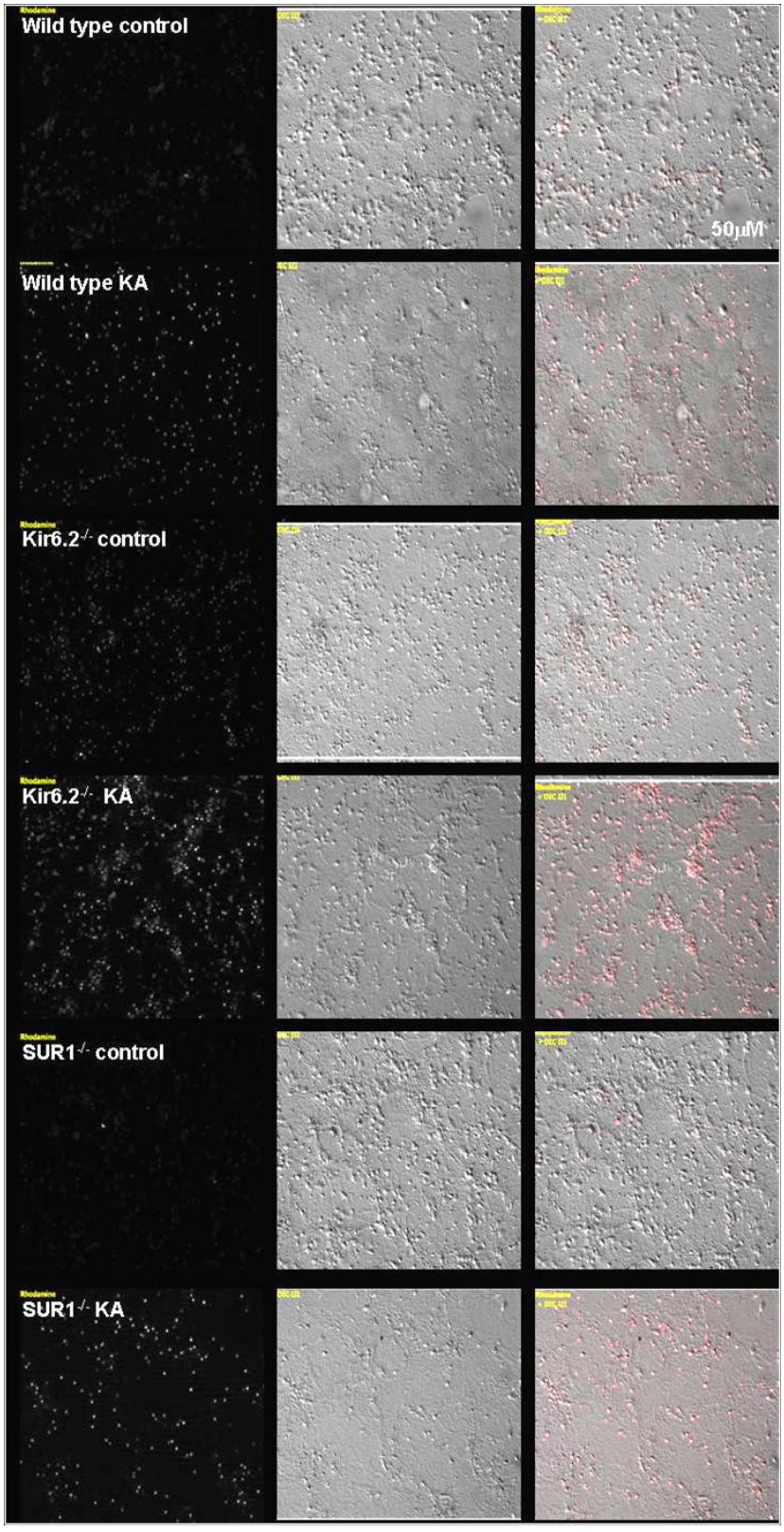


Figure 13. Neurons in mutant mice with K_{ATP} channel deficiencies are vulnerable to KA (LDH release)

Cortical neuron cultures (DIV14) from wild type, Kir6.2$^{-/-}$, and SUR1$^{-/-}$ mice were exposed to 400 μM kainic acid (KA) for one hour. Neuronal cell death was calculated by measuring the efflux of LDH 6 hrs later. Data is expressed as % LDH release by KA normalized to 500μM glutamate treatment (defined as 100%). Cultures from SUR$^{-/-}$ mice show increased neuronal cell death compared to wild type cultures (*$p < 0.005$, n = 4). This data is in concordance with the observations that absence of functional K_{ATP} channels increases glutamate receptor mediated excitotoxicity.

We observed similar results when kainic acid treated neurons were stained with Propidium Iodide (PI) (Figure 14). The SUR1$^{-/-}$ and Kir6.2$^{-/-}$ neurons showed increased PI staining compared to the wild type controls.

a Red DIC Overlap



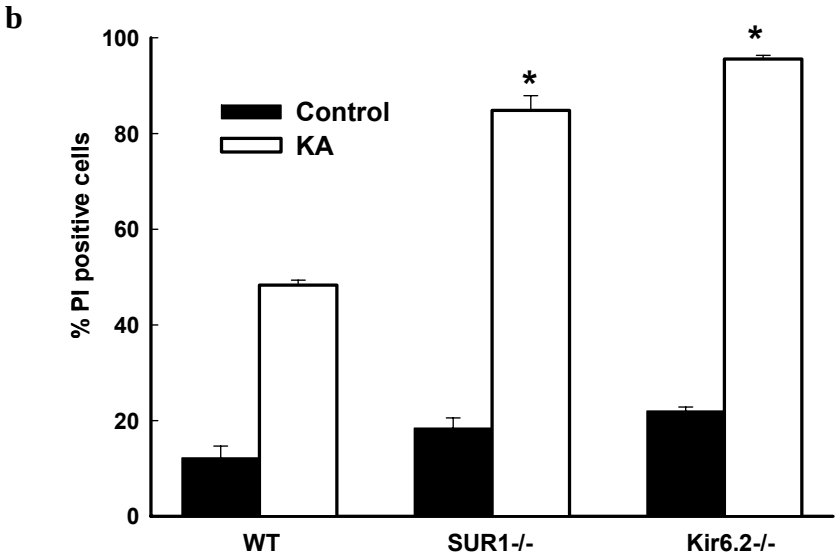


Figure 14. Neurons in mutant mice with K_{ATP} channel deficiencies are vulnerable to KA (PI staining)

Cortical neuron cultures (DIV14) from wild type, Kir6.1$^{-/-}$, and SUR1$^{-/-}$ mice were exposed to 400 μM kainic acid (KA) for one hour. a) Representative pictures of PI staining to show increased staining in SUR1$^{-/-}$ cultures compared to wild type controls. b) Neuronal cell death was quantified by counting PI positive cells. Data is expressed as percentage of PI positive cells over total number of cells. Cultures from SUR$^{-/-}$ and Kir6.2$^{-/-}$ mice show increased neuronal cell death compared to wild type cultures (*$p < 0.005$, $n = 4$).

Conclusion

- This study has identified for the first time the presence of Kir6.1 subunit in the active zone of the pre-synaptic terminal.
- This study also places Kir6.1/SUR1 type K_{ATP} channels and Syntaxin 1A in the same complex demonstrating their function in glutamate vesicle release.
- This data also means that Kir6.1 and SUR1 constitute the functional K_{ATP} channels in the active zone.
- Accordingly, Kir6.1/SUR1 type K_{ATP} channels in the hippocampus can block glutamate release thus curbing epileptogenesis.
- Finally, the results show that K_{ATP} channel deficient mice are more prone to neuronal cell death due to their inability to block glutamate-mediated excitotoxicity.

Discussion

To date, the only molecularly characterized K_{ATP} channel components expressed in the brain regions including the hypothalamus [47], basal forebrain cholinergic neurons [48], and striatum [49], is the Kir6.2 subunits, which are thought to protect against hypoxic insults. Although pharmacological approaches suggest the presence of SUR1 receptors [116], their functional properties in the brain have yet to be fully characterized. In the present study, we used electron microscopy combined with gene targeting studies. Our results clearly demonstrate that, as opposed to the Kir6.2-containing channels, functional K_{ATP} channels at pre-synaptic terminals in the hippocampus are formed by Kir6.1 subunits and SUR1 receptors. Mice with deficiencies in expression of either the SUR1 gene or the Kir6.1 gene are vulnerable to KA-induced seizures, an animal model of human temporal lobe epilepsy [9]. Thus, expression of functional Kir6.1/SUR1 channels can be considered as an endogenous cellular protective event against epileptic activity.

Behaviorally observable seizures are induced by sustained, paroxysmal neuronal network activity due to the spreading of uncontrolled excessive glutamate synaptic transmission in the hippocampus [9]. Consistent with this, our data show that release probability of glutamate at CA3 synapses lacking the Kir6.1/SUR1 channels, but not the Kir6.2-containing channels, is increased during induction of epileptic form of seizures. Hence, expression of functional Kir6.1/SUR1 channels is able to attenuate the danger of over-stimulation of glutamate transmission for generation of epileptic form of seizures.

The release of glutamate by pre-synaptic terminals is the initial event in generation of epileptic activity. The link between KA receptor activation and glutamate release is vague. Rise in intracellular Ca^{2+} concentration is a trigger for secretion as seen in insulin granules in the

pancreatic β-cells [63] and glutamate vesicle release in neuronal cells [117]. In the presence of glucose, high concentrations of ATP inhibit the K_{ATP} channels. This depolarizes β-cell membranes opening voltage-gated Ca^{2+} channels (VDCC) [44]. In pancreatic β-cells, Epac2, a cAMP dependent Guanine exchange factor interacts with the NB-1 domain of the SUR1 subunit. This interaction inhibits the K_{ATP} channel and increases insulin secretion.

Similar to insulin secretion from β cells, glutamate release from pre-synaptic terminals is triggered by Ca^{2+} influx through VDCC, which is known to be regulated by Epac2 protein complex including Rim2 and Piccolo. Piccolo in addition can bind to the SUR1 receptors [118-120]. It has been known that activation of KA receptors elevates cAMP levels [121];[122, 123]. Thus, application of KA may activate cAMP-dependent Epac2 in the Piccolo/SUR1/Kir6.1 channel complex and in turn regulate Ca^{2+}-dependent glutamate release.

CHAPTER II
AMPA RECEPTOR SUBUNIT GLUR2 GATES INJURIOUS SIGNALS IN ISCHEMIC STROKE

Back Ground

Ischemic stroke

Ischemic stroke is caused by occlusion of blood vessels supplying the brain and is the third leading cause of death in developed countries. A critical feature of brain injury following transient forebrain ischemic insult is a highly selective pattern of neuronal loss in the central nervous system (CNS). Certain identifiable subsets of neurons are severely damaged, including CA1 pyramidal neurons in the hippocampus, cortical projection neurons in layer 3, and medium spiny neurons in the dorsolateral striatum, whereas other neurons in the brain remain intact [32, 113, 124]. One step in this selective neuronal injury involves excessive stimulation of glutamate receptor (GluR) channels, allowing inflow of massive amounts of Ca^{2+}, Zn^{2+}, and Na^{+} in vulnerable neurons [125]. Ca^{2+} overload can trigger several downstream lethal reactions, including nitrosative stress [126-130], oxidative stress [131, 132], and mitochondrial dysfunction[133-136], as reviewed earlier[125, 137] and represented in figure 15. The over stimulation of GluRs is the primary intracellular event that induces neuronal death. Therefore, synaptic GluR channels have been considered a promising target for stroke therapy [138].

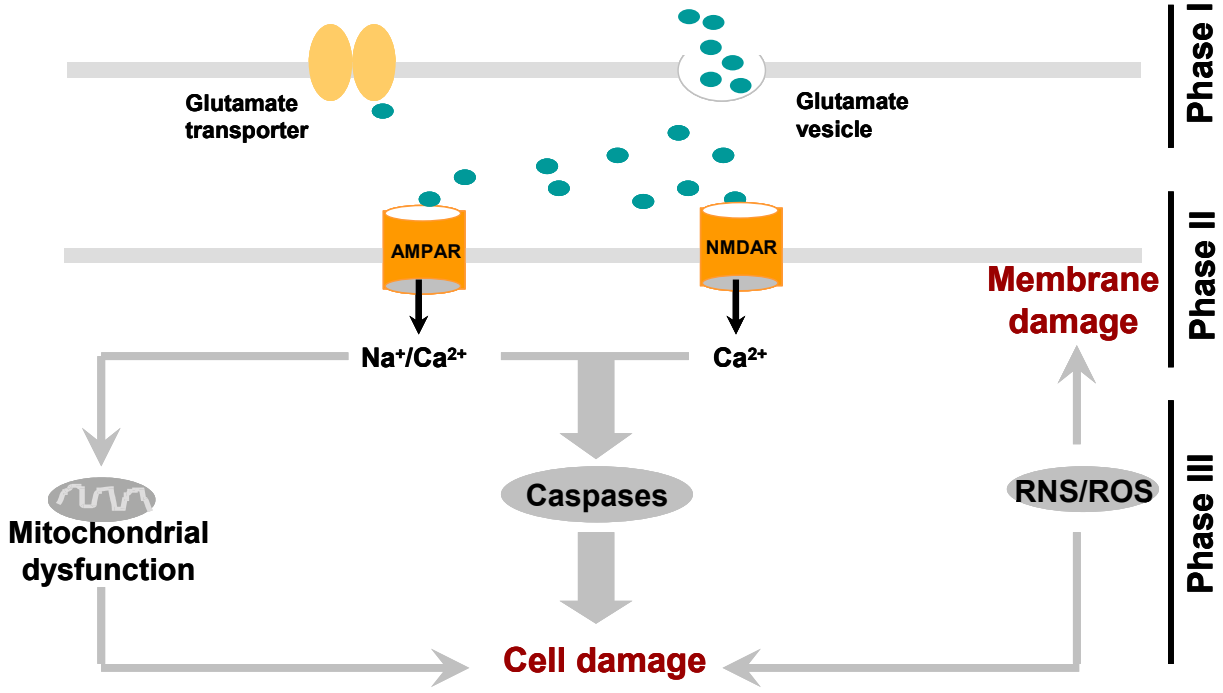


Figure 15. Glutamate toxicity in ischemic injury.

CA1 pyramidal neurons degenerate 3 to 6 days after transient forebrain ischemia. The cellular events underlying this delayed neuronal injury involve three phases. **Phase I**: during an episode (approx 5 min) of forebrain ischemia, extracellular glutamate concentration in CA1 area of the hippocampus increases because of increased vesicle release or decreased re-uptake by the glutamate transporters. Ten minutes after ischemia, glutamate returns to the basal levels. **Phase II**: occurs 6 to 12 h after ischemia. A critical feature in this phase is the sustained changes of glutamate receptor channel activity, including: AMPA receptor channels become permeable to Ca^{2+} and NMDA receptor channel activity is enhanced. Subsequently, sustained Ca^{2+} inflow leads to mitochondria dysfunction and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). **Phase III**: about 48 h after ischemia, ultimately, the neuronal cell is damaged.

Glutamate toxicity in neuronal injury

Glutamate plays an essential role in neural development, excitatory synaptic transmission, and plasticity [24, 139-143]. Immediately following ischemia, however, glutamate accumulates at synapses [144-146], resulting in extensive stimulation of its receptors, which can eventually be neurotoxic [125, 137, 147, 148]. Glutamate activates three classes of ionophore-linked postsynaptic receptors—namely, NMDA, AMPA and kainic acid receptors. NMDA receptor toxicity is dependent on extracellular Ca^{2+} and therefore, may reflect a large amount of Ca^{2+} influx directly through the receptor-gated ion channels [125, 137, 147-150]. Because most AMPA receptor channels have poor Ca^{2+} permeability (with exceptions, as discussed later), injury may result primarily from indirect Ca^{2+} entry through Ca^{2+}-permeable transient receptor potential channel member (TRPM)-7 channels[127], Ca^{2+}- permeable acid-sensing ion channels [151], and voltage-gated Ca^{2+} channels [152]. Although excess stimulation of GluRs contributes to ischemic brain injury, blocking them completely could be deleterious to animals and humans because targeting these receptors would also block the physiological action of glutamate in noninjured neurons. Therefore, it may be fruitful to develop a practical approach whereby the pathological effects of glutamate in vulnerable neurons is selectively blocked, leaving the physiological action of glutamate in the CNS unaffected.

NMDA receptor channels in selective neuronal injury

Glutamate toxicity largely results from NMDA receptor stimulation [125, 126, 137, 147, 148, 153]. Knockout mice lacking the NMDA receptor *NR2A* gene show decreased cortical infarction after focal cerebral ischemia [154]. Systemic administration of an NMDA receptor antagonist protects CA1 pyramidal neurons from ischemic injury [143], suggesting that activation of NMDA receptor channels following ischemia may mediate selective neuronal injury. However, these channels are widely expressed in many types of neurons, including ischemia-sensitive and -insensitive neurons in the CNS [155, 156]. Therefore, certain pathological signaling molecules that modulate NMDA receptor function in vulnerable CA1 neurons may contribute to selective neuronal loss in the hippocampus of rats following transient forebrain ischemia [113]. NMDA receptor channels are heteromeric complexes consisting of an essential NR1 subunit and one or more regulatory NR2 subunits, NR2A-D [24, 155, 156]. It was recently discovered that transient forebrain ischemia causes phosphorylation of NR2A at serine-1232 (phospho- Ser1232) in rat CA1 neurons in vivo [113]. It was also determined that cyclin-dependent kinase 5 (Cdk5) catalyzes Ser1232 phosphorylation and that expression of a C-terminal peptide fragment of NR2A inhibits endogenous Cdk5 (or perturbs the Cdk5-NR2A interaction), thereby abolishing Ser1232 phosphorylation and protecting CA1 pyramidal neurons from ischemic insult [113]. In addition, Cdk5-mediated regulation of NMDA receptor channels occurs only in CA1 neurons and not in other areas of the hippocampus. This shows that the covalent modification of NMDA receptors by Cdk5 is an essential intracellular event that determines vulnerability of neurons to the insult [113]. Therefore, endogenous Cdk5 can be considered a suitable target regarding modification of cell survival during disease.

Expression of Ca^{2+}-Permeable AMPA receptors in vulnerable CA1 pyramidal neurons

Activation of endogenous Cdk5 depends on Ca^{2+} entry through AMPA receptors [113], which constitute a major GluR subtype [139, 141, 142, 144, 146]. Functional AMPA receptor channels are assembled from GluR subunits 1 through 4 [139, 141, 142, 144, 146]. The Ca^{2+} permeability of AMPA receptor channels is determined by the GluR2 subunit [28, 29, 157-162]. In the adult CNS, nearly 100% of the messenger RNA (mRNA) encoding GluR2 is edited at the Q/R site corresponding to residue 607, where the genomic glutamine (Q607) codon is converted to an arginine (R) codon (Figure 16) [28, 157-159, 161, 162]. Edited GluR2(R) subunits form Ca^{2+}-impermeable channels, whereas unedited GluR2(Q) channels allow Ca^{2+} entry [28, 29, 157-162] (Figure 16).

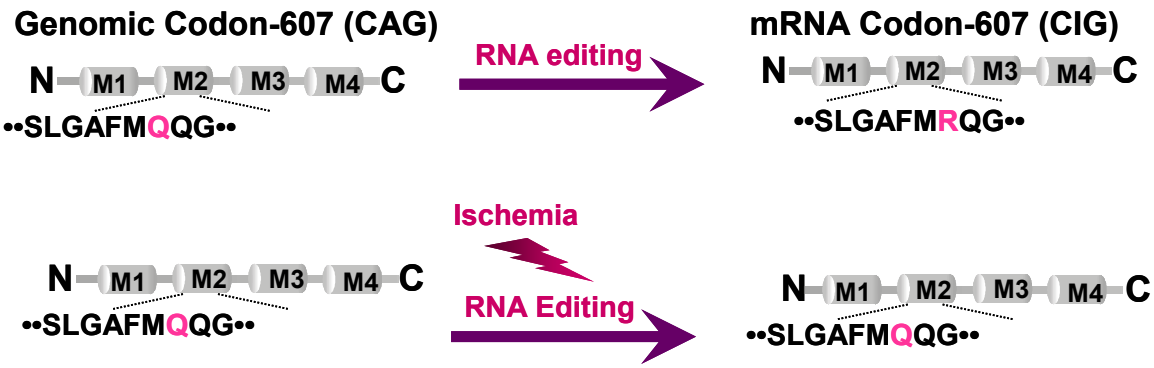


Figure 16. GluR2 gene is edited at the Q/R site

GluR2 gene is edited at the genomic glutamine codon (Q607). This changes the aminoacid to an arginine forming Ca^{2+} impermeable GluR2(R) AMPA channels. During ischemia, this RNA editing is disrupted causing increased expression of GluR2(Q) AMPA channels that are permeable to Ca^{2+}. This Ca^{2+} leads to excitotoxicity and cell death.

In most CA1 neurons, AMPA receptor channels contain the GluR2(R) subunits and, therefore, are impermeable to Ca^{2+} entry [29]. A recent study showed that transient forebrain ischemia reduces GluR2 mRNA levels in hippocampal CA1 neurons [110]. This observation led to the "GluR2 hypothesis," which postulates that reduced GluR2 expression allows Ca^{2+} entry through AMPA receptor channels, which, in turn, induces CA1 pyramidal cell death [110, 163-165]. The GluR2 hypothesis was proposed based on a causal relationship between CA1 pyramidal cell injury and reduced *GluR2* gene expression following transient forebrain ischemia [164, 165]. Therefore, it is important to understand whether a decrease of GluR2 mRNA actually causes AMPA receptor channels in vulnerable CA1 pramidal neurons to become permeable to Ca^{2+} entry. Ca^{2+} indicator dyes can be used to estimate AMPA receptor-mediated Ca^{2+} transients in CA1 pyramidal neurons. Indeed, an earlier work demonstrated that AMPA caused a large rise of intracellular Ca^{2+} in postischemic neurons compared to nonischemic control neurons [164]. Recent studies involving whole-cell patch clamp recordings in individual CA1 pyramidal neurons from the rat hippocampus (visualized with infrared illumination and differential interference contrast (DIC, infrared- DIC) optics systems) [113 32] showed that AMPA receptors containing GluR2(R) exhibit inward currents with high extra cellular Na^{+}, but not high Ca^{2+}. In contrast receptors lacking GluR2(R) showed large inward currents with either high Na^{+} or Ca^{2+} [32]. These data allowed for the determination of the permeability of AMPA receptor channels to Ca^{2+} (PCa/PNa) by calculating the shift in reversal potentials of AMPA receptor-mediated excitatory postsynaptic currents ($EPSCs_{AMPA}$) when the extra cellular solution was switched from high Na^{+} to high Ca^{2+}. Using this electrophysiological approach, it was also estimated that the Ca^{2+} permeability of AMPA receptor channels in CA1 pyramidal neurons of rats 12 h after transient global ischemia was 18-fold greater than that in sham controls. These data demonstrate that transient forebrain

ischemia induces AMPA receptor channels permeable to Ca^{2+} entry into vulnerable CA1 pyramidal cells.

Transcriptional regulation of GluR2 expression in CA1 neurons

Cell-survival-related gene expression has its basis in signaling cascades initiated by the transcription factor Cyclic adenine monophospate Response Element Binding protein (CREB) [166-169], which is believed to regulate GluR2 promoter activity [170]. Therefore, CREB activation must stimulate the expression of the *GluR2* gene and in turn, inhibit GluR2- gated injurious signals in vulnerable CA1 neurons [32]. Accordingly, when a constitutively active CREB, VP16-CREB, was expressed in the rat CA1 pyramidal neurons in vivo [32] it inhibited Ca^{2+}-permeable AMPA receptor channels in vulnerable CA1 pyramidal neurons following transient forebrain ischemia. Although the exact nature of the CREB signals for neuronal survival remains to be determined, expression of GluR2(R) may be one of the CREB pathways responsible for neuronal survival, because the protective role of CREB is abolished by directly introducing unedited GluR2(Q) gene [32]. Therefore, on one hand, CREB increases GluR2 gene expression, thereby increasing the relative level of GluR2(R). The finding that CREB induces expression of brain-derived neurotrophic factor (BDNF) supports this hypothesis [171, 172]. BDNF binds to the GluR2 promoter that contains the gene silencing transcription factor neuronal repressor element-1 silencing transcription factor (REST)/neuron-restrictive silencer element (NRSE). Additionally, it was reported that forebrain ischemia induces expression of REST and, in turn, reduces GluR2 gene expression [173]. Importantly, acute knockdown of the REST gene rescues postischemic neurons from ischemia-induced cell death [173]. Therefore, BDNF probably disrupts the interaction

of REST/NRSE with the GluR2 promoter, thus stimulating GluR2 gene expression [170]. On the other hand, CREB activation may directly facilitate Q/R site editing of GluR2 mRNA, because increased mRNA editing also increases GluR2 mRNA levels by reducing nuclear accumulation of incompletely processed primary transcripts [174].

Inhibition of Ca^{2+}-permeable AMPA receptor channels protects vulnerable neurons from forebrain ischemic insult

Transient forebrain ischemia induces expression of Ca^{2+}-permeable AMPA receptor channels in vulnerable CA1 neurons, which then activates a series of intracellular injurious signals [113]. To investigate this possibility, it is necessary to selectively block Ca^{2+} permeability of AMPA receptor channels in rat CA1 vulnerable neurons *in vivo*. An early work reported that administration of Naspm, an open-channel blocker selective for Ca^{2+}-permeable AMPA receptor [175, 176], reduced GluR2 antisense-induced cell death and suggested that cell death induced by multiple injections of GluR2 antisense was mediated by Ca^{2+}-permeable AMPA receptors [163]. Recently, virus-based gene expression vectors was used to introduce the *GluR2(R)* gene that is independent of Q/R site editing into the adult rat hippocampus in vivo. Using whole-cell patch clamp recordings, Liu S et al., obtained direct evidence that introduction of the *GluR2(R)* gene selectively inhibits Ca^{2+} permeability of AMPA receptor channels occurring in vulnerable CA1 pyramidal neurons of adult rats following transient forebrain ischemia [32]. This study provides the first direct evidence that selective inhibition of Ca^{2+} permeability of AMPA receptor channels rescues CA1 pyramidal neurons from forebrain ischemic insult [32].

In contrast to CA1 pyramidal neurons, granule neurons in the hippocampus are insensitive to ischemic injury [32, 113, 124], and AMPA receptor channels in these cells are Ca^{2+}-

impermeable. To further demonstrate whether expression of Ca^{2+}-permeable AMPA receptor channels determine vulnerability of neurons to the insult, the granule cells were engineered to express Ca^{2+}-permeable GluR2(Q) channels by direct introduction of exogenous unedited *GluR2(Q)* gene into the adult hippocampus. Following this manipulation, it was found that the granule neurons degenerate following transient global ischemia [32]. Similarly to these results observed upon expression of unedited *GluR2(Q)* gene, a recent report showed that delivery of GluR2(Q) complementary DNA into the hippocampus by a liposome-mediated gene transfer increases the sensitivity of neurons in the hippocampus to forebrain ischemia insult [177]. Together, it is suggested that the expression of Ca^{2+}-permeable AMPA receptor channels provides a route for toxic Ca^{2+} entry, which then triggers neuronal cell death in the hippocampus [178-182]. Ca^{2+}-permeable AMPA receptor channels are not only permeable to Ca^{2+} but are also highly permeable to Zn^{2+} [178-182] and that a brief ischemic insult in rats induces Zn^{2+} accumulation in vulnerable neurons [183, 184]. Therefore, Ca^{2+}/Zn^{2+} influx can be considered an initial event for activation of an array of downstream enzymes that degrade membranes and proteins essential for cellular integrity, as illustrated in Fig. 15. Thus, recent results demonstrate that expression of the *GluR2(R)* gene blocks Ca^{2+} entry through AMPA receptor channels and prevents activation of endogenous Cdk5 in vulnerable CA1 neurons [32, 113]. Furthermore, Cdk5 activation causes the sustained opening of NMDA receptor channels, resulting in an intracellular Ca^{2+} overload. Taken together these date leads to a model where GluR2-gated intracellular signaling cascade determines selective neuronal vulnerability after forebrain ischemia, as described in Fig. 17.

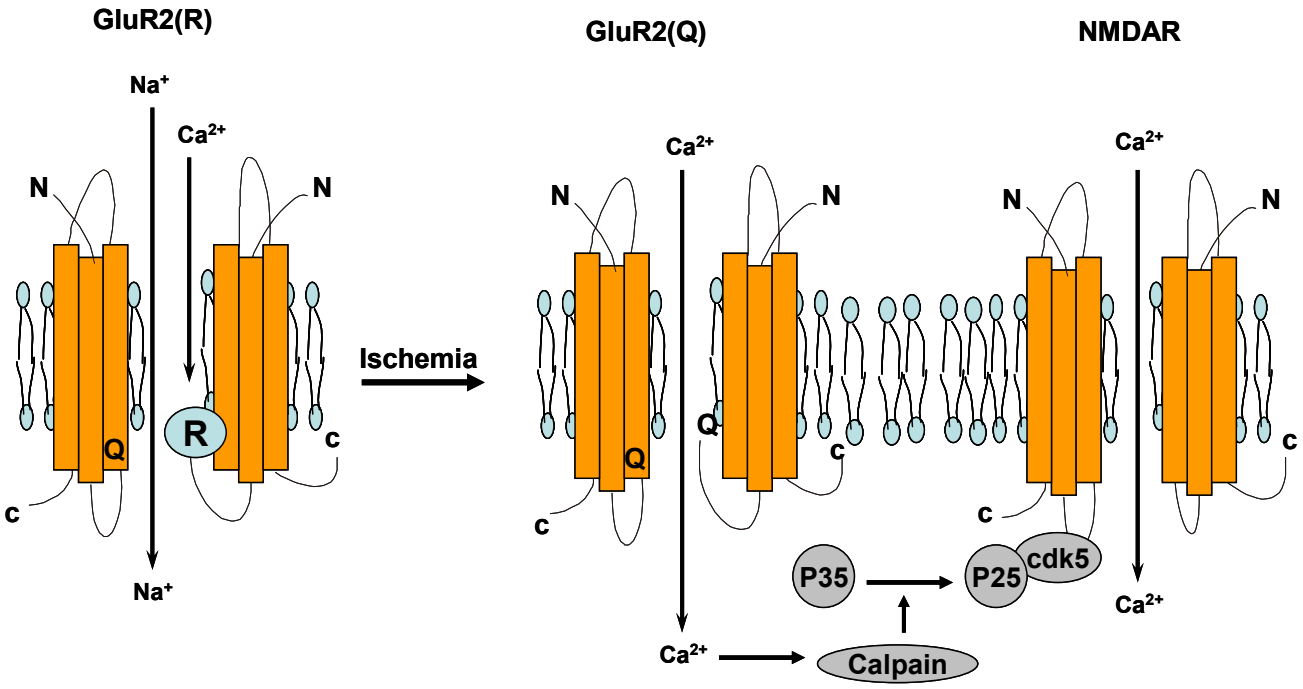


Figure 17. Induction of Ca^{2+}-permeable AMPA receptor channels primes cell death.

AMPA receptor channels in CA1 pyramidal neurons normally contain an edited form of GluR2 subunit (GluR2[R]) and, therefore, are Ca^{2+} impermeable. Following transient forebrain ischemia, GluR2(R) levels in CA1 neurons decreases, resulting in Ca^{2+} entry through AMPA receptor channels. The subsequent p25 accumulation leads to prolonged activation of Cdk5, which phosphorylates the NR2A subunit of NMDA receptors at Ser1232 and induces further Ca^{2+} entry through NMDA receptor channels, leading to cell death.

Hypothesis
Ca^{2+} permeable AMPA channel expression is increased during transient forebrain ischemia

Delivery of GluR2 proteins from intracellular pools to the cell surface is required for expression of functional AMPA receptor channels at CA1 synapses[184, 185] as illustrated in Fig. 18.. So here we hypothesize that reduced GluR2 expression during an ischemic insult will reduce the expression of functional AMPA channels on the cell surface. To test this hypothesis, we performed electrophysiological analysis on CA1 synapses during an ischemic insult. We also compared the expression of GluR2 protein levels between ischemic and physiological conditions.

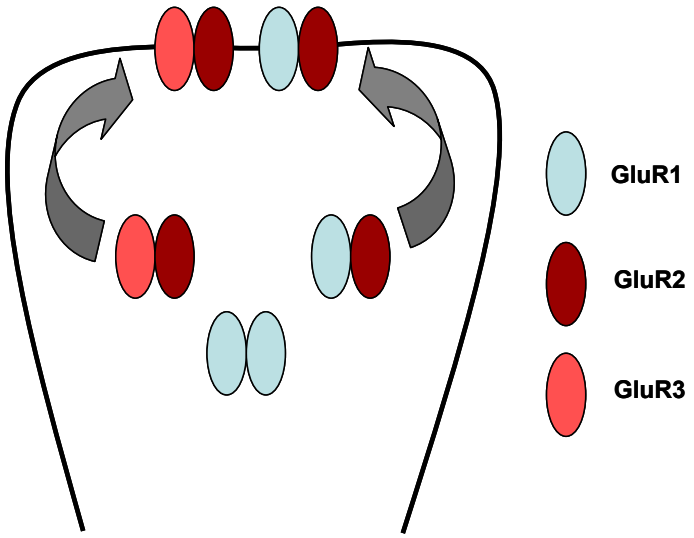


Figure 18. Surface expression of GluR2 subunit is increased during ischemia

The illustration shows GluR2-dependent translocation of GluR1/GluR2 receptor channels at CA1 synapses. GluR1 is unable to incorporate into cell surface without GluR2.

Materials and methods

Transient forebrain ischemia

Transient forebrain ischemia was induced in rats as described before [31]. Briefly, 80 ± 5-day-old Sprague Dawley rats were used. The bilateral vertebral arteries were exposed, coagulated and cut completely 12 hr before the induction of ischemia. Ischemia was induced by bilaterally occluding the common carotid arteries with aneurysm clips for 15 mins, after which the clips were removed to restore cerebral blood flow. Body temperature was maintained at 37ºC with heating pads until the animals had recovered from surgery. Sham-operated animals were treated identically, except that the carotid arteries were not occluded. The brain was extracted from these animals 12h later and processed for electrophysiology recordings or protein extraction for immunoblotting analysis.

Electrophysiology

Sample traces of miniature $EPSCs_{AMPA}$ taken from CA1 pyramidal neurons in hippocampal slices from rats 12 h after sham or transient forebrain ischemia. Hippocampal slices (250 μm) were prepared from mice, as described before [31, 32, 113]. Slices in the recording chamber were continuously perfused with artificial cerebrospinal fluid (ACSF, 2 ml/min) saturated with 95% O_2/5% CO_2 at 30°C ± 1°C. The composition of ACSF was 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM dextrose. For whole-cell patch-clamp recordings (tight seal >15-25 GΩ) from CA3 pyramidal cells, hippocampal slices were visualized with IR-DIC optics with an Axioskop 2FS equipped with Hamamatsu

C2400-07E optics. The spontaneous EPSCs were recorded at a holding potential of -70 mV. The intracellular solution contained 142.5 mM Cs-gluconate, 7.5 mM CsCl, 10 mM HEPES, 0.2 mM EGTA, 2 mM Mg-ATP, 0.3 mM guanosine triphosphate (pH 7.4; 296 mOsm), 50 μ*M* of AP5, 10 μ*M* of Bicucculline. The currents were filtered at 5 kHz with a low-pass filter. Data were digitized at a frequency of 10 kHz and stored online with the pclamp11 system (Axon Instruments, Inc., Foster, CA). The input resistance and series resistance in postsynaptic pyramidal cells were monitored with prevoltage steps (−2 mV; 100 ms) at 5 min intervals throughout the period of the experiment. Series resistance ranged from 11 to 17 MΩ. Input resistance was 346 ± 18 MΩ. Data are mean ± SEM (*n* = 8 cells/four animals)

Detection of GluR2 protein levels

The CA1 extracts (20 μg of protein) was obtained from rats 6h and 12h after they underwent transient forebrain ischemia or sham. It was denatured with sodium dodecyl sulfate sample buffer, separated by SDS-PolyAcrylamide Gel Electrophoresis on a 12% gel, transferred into nitrocellulose membranes, and incubated with rabbit anti-GluR1 (1:500; Chemicon), anti-GluR2 (1:200; Santa Cruz), anti-GluR3 (1: 200; Santa Cruz), antiGluR4 (1:500; Santa Cruz) and anti-α-actin (1:1000; Chemicon). Immunoreactive bands were detected using an enhanced Chemiluminescent kit (Amersham Biosciences) and quantified using Densitometer Quantity One (Bio-Rad). Each reactive band was normalized to its respective sham (defined as 1.0). Data are mean ± SEM (n = 4; *p < 0.01, compared to sham).

Results

The numbers of functional AMPA channels do not change during ischemia

The electrophysiological recordings of the miniature $EPSCs_{AMPA}$ from CA1 pyramidal neurons revealed that the mean amplitude of the miniature $EPSCs_{AMPA}$ in rats that were subjected to transient forebrain ischemia was the same as that in sham animals (Fig. 19 a and b). These results are consistent with previous reports that, although AMPA receptor channels are permeable to Ca^{2+} [32, 113, 186], the $EPSCs_{AMPA}$ are not reduced [187, 188]. Because two distinct GluR1/GluR2 and GluR3/GluR2 channels are located in CA1 pyramidal neurons [189-191], unchanged amplitudes of synaptic responses following reduced *GluR2* gene expression could result from over expression of GluR1 and GluR3 subunits.

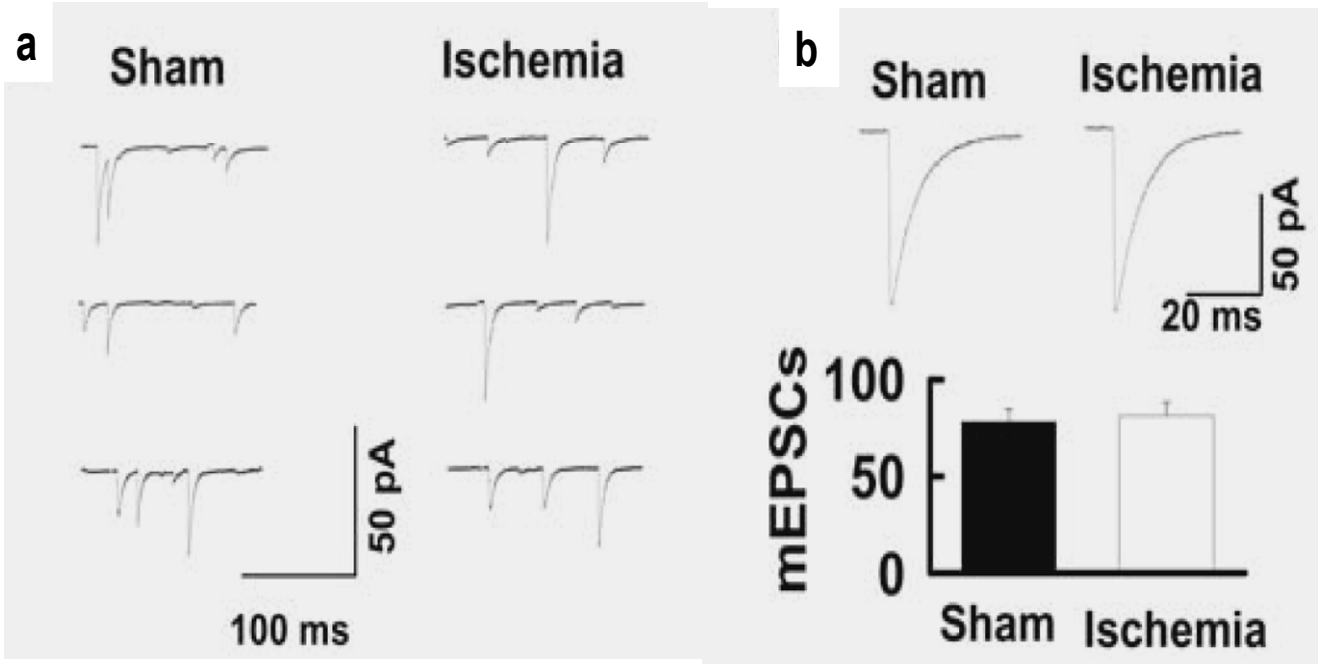


Figure 19. The mean amplitude of miniature EPSCs$_{AMPA}$ remain unchanged in CA1 pyramidal neurons following transient forebrain ischemia.

a) Sample traces of miniature EPSCs$_{AMPA}$ taken from CA1 pyramidal neurons in hippocampal slices from rats 12 h after sham or transient forebrain ischemia. b) Averaged traces of the miniature EPSCs$_{AMPA}$ from (a) are shown on the top. The mean amplitudes (pA) of the responses are summarized in bar graph.

GluR2 protein level is reduced by transient forebrain ischemia

We analyzed protein levels of AMPA receptor subunits in CA1 area of the hippocampus from rats 12 h after transient forebrain ischemia. Western blots demonstrated that the GluR2 proteins in CA1 dorsal hippocampus were reduced by 38.8 ± 4.9% (n = 6; Fig. 20) compared to sham control rats. Unlike the GluR2 subunit, protein levels of GluR1, GluR3, and GluR4 subunits in CA1 neurons following transient forebrain ischemia did not show a significant change.

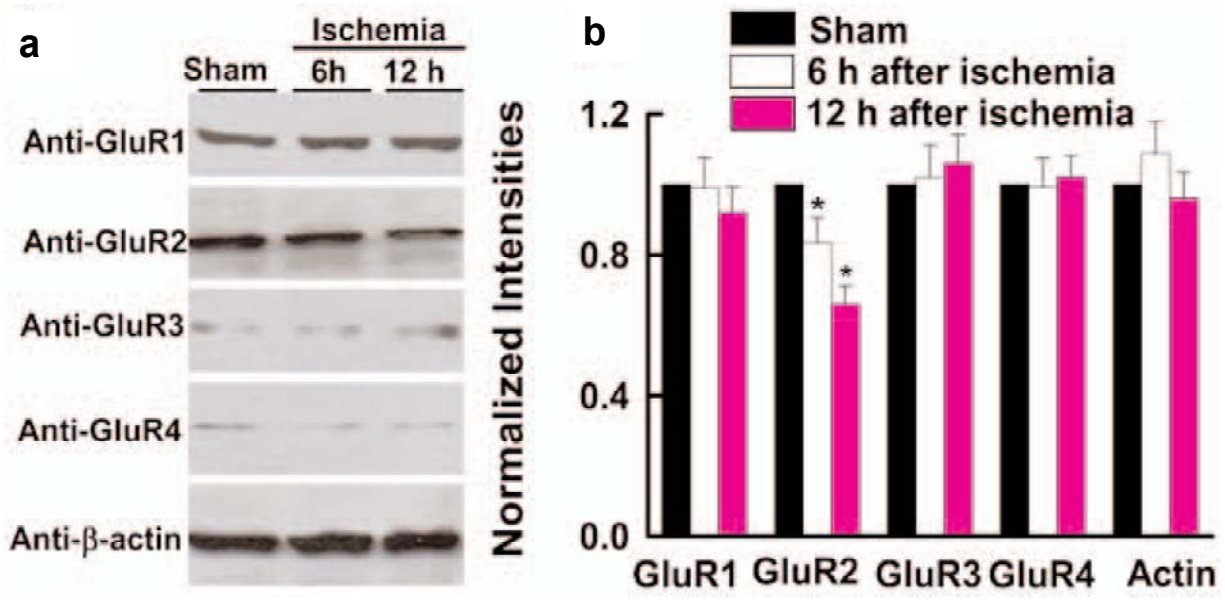


Figure 20. GluR2 proteins in CA1 hippocampus is reduced following transient forebrain ischemia.

a) Representative Western blots of the CA1 cell lysates blotted with the indicated antibodies. b) Summarized data are shown in the bar graph. (*$p < 0.01$)

Conclusion

- The GluR2 subunit is a requisite for AMPA channel expression on the neuronal cell surface. Hence, reduced GluR2 protein levels mean less number of channels on the cell surface. However, this study shows that the amplitude of current flowing through the AMPA receptors does not change during transient ischemia. This means the number of functional AMPA channels do not change because of the ischemic insult.
- Previous data shows that when GluR2 Q/R site editing is impaired the unedited transcripts accumulate in the nucleus reducing expressed GluR2 protein levels. Also, the Ca^{2+}/Zn^{2+} permeability of AMPA receptor channels is blocked by GluR2(R) Ca^{2+}-impermeable AMPA receptor channels, whereas the unedited GluR2(Q) allows Ca^{2+} entry through the channels. This study also shows that transient forebrain ischemia reduces GluR2 protein levels. As the number of functional AMPA channels on the cell surface is not reduced, we suggest that transient forebrain ischemia impairs GluR2 pre-mRNA editing hence decreasing the number of unedited GluR2 transcripts and total GluR2 protein levels. This increases the number of GluR2 (Q) channels on the cell surface and in turn will increase the amount of Ca^{2+} entry into vulnerable neurons.

Discussion

The Ca^{2+}/Zn^{2+} permeability of AMPA receptor channels is dominated by the GluR2 subunit; an edited GluR2(R) forms Ca^{2+}-impermeable AMPA receptor channels, whereas the unedited GluR2(Q) allows Ca^{2+} entry through the channels [28, 29, 157-159, 161, 162]. Accordingly, expression of Ca^{2+}/Zn^{2+}-permeable AMPA receptor channels in vulnerable CA1 neurons following transient forebrain ischemia can simply result from reduced *GluR2* gene expression, as described earlier. Notably, delivery of GluR2 proteins from intracellular pools to the cell surface is required for expression of functional AMPA receptor channels at CA1 synapses[184, 185]. Therefore, reduction of GluR2 protein levels should decrease the numbers of functional AMPA receptor channels, as illustrated in Fig. 18a.

However, electrophysiological recordings of the miniature $EPSCs_{AMPA}$ from CA1 pyramidal neurons revealed that the mean amplitude of the miniature $EPSCs_{AMPA}$ in rats that were subjected to transient forebrain ischemia was the same as that in sham animals (Fig. 18 b and c). These results are consistent with previous reports that, although AMPA receptor channels are permeable to Ca^{2+} [32, 113], the $EPSCs_{AMPA}$ are not reduced [187, 188]. Because two distinct GluR1/GluR2 and GluR3/GluR2 channels are located in CA1 pyramidal neurons [189-191], unchanged amplitudes of synaptic responses following reduced *GluR2* gene expression could result from over expression of GluR1 and GluR3 subunits.

To address this question, we analyzed protein levels of AMPA receptor subunits in CA1 area of the hippocampus from rats after transient forebrain ischemia. Western blots demonstrated that the GluR2 proteins in CA1 dorsal hippocampus were reduced while the GluR1, GluR3, and GluR4 subunits protein levels was not changed. Together, we suggest that transient forebrain

ischemia facilitates the events for incorporation of GluR2 subunits to plasma membrane. Under this circumstance, although *GluR2* gene expression is reduced, the numbers of functional AMPA receptor channels remain unchanged.

Previous studies revealed that Q/R site unedited GluR2 easily exits the endoplasmic reticulum and becomes the cell surface receptor channels [192, 193]. These findings implicate that pre-mRNA editing of GluR2 at Q/R site could be impaired in vulnerable CA1 pyramidal neurons following transient forebrain ischemia. Consistent with this hypothesis, recent studies have demonstrated that impaired Q/R site editing contributes to selective motor neuronal death in patients with amyotrophic lateral sclerosis [194, 195]. Gene-targeting studies demonstrated that the deficits of GluR2 pre-RNA editing at Q/R site reduced GluR2 mRNA levels because of nuclear accumulation of incompletely processed primary GluR2 transcripts [185]. Therefore, reduced GluR2 mRNA levels observed in the CA1 area of the rat hippocampus following transient forebrain ischemia may result from reduced Q/R site editing of GluR2 pre-mRNA. To address this question, it is necessary to use single-cell reverse transcriptase polymerase chain reaction to estimate GluR2 Q/R editing versus pre-mRNA accumulation in the nucleus of individual neurons in the rat hippocampus.

To date, all clinical stroke trials targeting GluRs (AMPA or NMDA) have failed, possibly because receptor antagonists also block the physiological actions of glutamate in uninjured neurons [196, 197]. This work outlines a potential approach to selectively block the pathological effects of AMPA receptors by targeting GluR2 subunit, thereby inhibiting only Ca^{2+}/Zn^{2+} permeability. Thus, this approach should not affect the physiological actions of Ca^{2+}/Zn^{2+}-impermeable AMPA receptors in uninjured neurons in the CNS. Therefore, this work may define a promising target for stroke therapy. As reported [194, 195], a defect in GluR2 editing also

contributes to motor neuron death in amyotrophic lateral sclerosis. It is probable that a similar therapeutic approach may apply to this disease.

REFERENCES

1. Annegers,J.F. Epidemiology and genetics of epilepsy. *Neurol. Clin.* **12**, 15-29 (1994).

2. Verrotti,A., Salladini,C., Di,M.G., Pisciella,F., & Chiarelli,F. Extended-release formulations in epilepsy. *J. Child Neurol.* **22**, 419-426 (2007).

3. Genton,P. & Bureau,M. Epilepsy with myoclonic absences. *CNS. Drugs* **20**, 911-916 (2006).

4. Kellinghaus,C. & Luders,H.O. Frontal lobe epilepsy. *Epileptic. Disord.* **6**, 223-239 (2004).

5. Liu,Y.W. *et al.* Adult neurogenesis in mesial temporal lobe epilepsy: a review of recent animal and human studies. *Curr. Pharm. Biotechnol.* **8**, 187-194 (2007).

6. Majores,M., Schoch,S., Lie,A., & Becker,A.J. Molecular neuropathology of temporal lobe epilepsy: complementary approaches in animal models and human disease tissue. *Epilepsia* **48 Suppl 2**, 4-12 (2007).

7. Naegele,J.R. Neuroprotective strategies to avert seizure-induced neurodegeneration in epilepsy. *Epilepsia* **48 Suppl 2**, 107-117 (2007).

8. Avoli,M., Louvel,J., Pumain,R., & Kohling,R. Cellular and molecular mechanisms of epilepsy in the human brain. *Prog. Neurobiol.* **77**, 166-200 (2005).

9. Ben-Ari,Y. & Cossart,R. Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci.* **23**, 580-587 (2000).

10. Magloczky,Z. & Freund,T.F. Impaired and repaired inhibitory circuits in the epileptic human hippocampus. *Trends Neurosci.* **28**, 334-340 (2005).

11. Bradford,H.F. Glutamate, GABA and epilepsy. *Prog. Neurobiol.* **47**, 477-511 (1995).

12. Sheng,M. & Kim,M.J. Postsynaptic signaling and plasticity mechanisms. *Science* **298**, 776-780 (2002).

13. Hara,M.R. & Snyder,S.H. Cell signaling and neuronal death. *Annu. Rev. Pharmacol. Toxicol.* **47**, 117-141 (2007).

14. Arundine,M. & Tymianski,M. Molecular mechanisms of glutamate-dependent neurodegeneration in ischemia and traumatic brain injury. *Cell Mol. Life Sci.* **61**, 657-668 (2004).

15. Yi,J.H. & Hazell,A.S. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochem. Int.* **48**, 394-403 (2006).

16. Meldrum,B.S. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J. Nutr.* **130**, 1007S-1015S (2000).

17. Schousboe,A. & Waagepetersen,H.S. Role of astrocytes in glutamate homeostasis: implications for excitotoxicity. *Neurotox. Res.* **8**, 221-225 (2005).

18. Camacho,A. & Massieu,L. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. *Arch. Med. Res.* **37**, 11-18 (2006).

19. Soundarapandian,M.M., Tu,W.H., Peng,P.L., Zervos,A.S., & Lu,Y. AMPA receptor subunit GluR2 gates injurious signals in ischemic stroke. *Mol. Neurobiol.* **32**, 145-155 (2005).

20. Collingridge,G.L. & Lester,R.A. Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.* **41**, 143-210 (1989).

21. Watkins,J.C. & Jane,D.E. The glutamate story. *Br. J. Pharmacol.* **147 Suppl 1**, S100-S108 (2006).

22. Birnbaumer,L. *et al.* The naming of voltage-gated calcium channels. *Neuron* **13**, 505-506 (1994).

23. Choi,D.W. Excitotoxic cell death. *J. Neurobiol.* **23**, 1261-1276 (1992).

24. Hollmann,M. & Heinemann,S. Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17**, 31-108 (1994).

25. Sucher,N.J., Awobuluyi,M., Choi,Y.B., & Lipton,S.A. NMDA receptors: from genes to channels. *Trends Pharmacol. Sci.* **17**, 348-355 (1996).

26. Choi,D.W. Ionic dependence of glutamate neurotoxicity. *J. Neurosci.* **7**, 369-379 (1987).

27. Cull-Candy,S., Kelly,L., & Farrant,M. Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond. *Curr. Opin. Neurobiol.* **16**, 288-297 (2006).

28. Jonas,P. & Burnashev,N. Molecular mechanisms controlling calcium entry through AMPA-type glutamate receptor channels. *Neuron* **15**, 987-990 (1995).

29. Geiger,J.R. *et al.* Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron* **15**, 193-204 (1995).

30. Pellegrini-Giampietro,D.E., Zukin,R.S., Bennett,M.V., Cho,S., & Pulsinelli,W.A. Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc. Natl. Acad. Sci. U. S. A* **89**, 10499-10503 (1992).

31. Peng,P.L. *et al.* ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. *Neuron* **49**, 719-733 (2006).

32. Liu,S. *et al.* Expression of Ca(2+)-permeable AMPA receptor channels primes cell death in transient forebrain ischemia. *Neuron* **43**, 43-55 (2004).

33. Lerma,J. Kainate receptor physiology. *Curr. Opin. Pharmacol.* **6**, 89-97 (2006).

34. Moldrich,R.X. & Beart,P.M. Emerging signalling and protein interactions mediated via metabotropic glutamate receptors. *Curr. Drug Targets. CNS. Neurol. Disord.* **2**, 109-122 (2003).

35. Nieoullon,A. *et al.* The neuronal excitatory amino acid transporter EAAC1/EAAT3: does it represent a major actor at the brain excitatory synapse? *J. Neurochem.* **98**, 1007-1018 (2006).

36. Moldrich,R.X., Chapman,A.G., De,S.G., & Meldrum,B.S. Glutamate metabotropic receptors as targets for drug therapy in epilepsy. *Eur. J. Pharmacol.* **476**, 3-16 (2003).

37. Nicoletti,F., Bruno,V., Copani,A., Casabona,G., & Knopfel,T. Metabotropic glutamate receptors: a new target for the therapy of neurodegenerative disorders? *Trends Neurosci.* **19**, 267-271 (1996).

38. Niswender,C.M., Jones,C.K., & Conn,P.J. New therapeutic frontiers for metabotropic glutamate receptors. *Curr. Top. Med. Chem.* **5**, 847-857 (2005).

39. Lawson,K. & McKay,N.G. Modulation of potassium channels as a therapeutic approach. *Curr. Pharm. Des* **12**, 459-470 (2006).

40. Ashcroft,F.M. & Gribble,F.M. Correlating structure and function in ATP-sensitive K+ channels. *Trends Neurosci.* **21**, 288-294 (1998).

41. Schwappach,B., Zerangue,N., Jan,Y.N., & Jan,L.Y. Molecular basis for K(ATP) assembly: transmembrane interactions mediate association of a K+ channel with an ABC transporter. *Neuron* **26**, 155-167 (2000).

42. Haider,S., Antcliff,J.F., Proks,P., Sansom,M.S., & Ashcroft,F.M. Focus on Kir6.2: a key component of the ATP-sensitive potassium channel. *J. Mol. Cell Cardiol.* **38**, 927-936 (2005).

43. Neagoe,I. & Schwappach,B. Pas de deux in groups of four--the biogenesis of KATP channels. *J. Mol. Cell Cardiol.* **38**, 887-894 (2005).

44. Nichols,C.G. KATP channels as molecular sensors of cellular metabolism. *Nature* **440**, 470-476 (2006).

45. Miki,T. & Seino,S. Roles of KATP channels as metabolic sensors in acute metabolic changes. *J. Mol. Cell Cardiol.* **38**, 917-925 (2005).

46. Kane,G.C., Liu,X.K., Yamada,S., Olson,T.M., & Terzic,A. Cardiac KATP channels in health and disease. *J. Mol. Cell Cardiol.* **38**, 937-943 (2005).

47. Miki,T. *et al.* ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. *Nat. Neurosci.* **4**, 507-512 (2001).

48. Allen,T.G. & Brown,D.A. Modulation of the excitability of cholinergic basal forebrain neurones by KATP channels. *J. Physiol* **554**, 353-370 (2004).

49. Avshalumov,M.V. & Rice,M.E. Activation of ATP-sensitive K+ (K(ATP)) channels by H2O2 underlies glutamate-dependent inhibition of striatal dopamine release. *Proc. Natl. Acad. Sci. U. S. A* **100**, 11729-11734 (2003).

50. Chen,M., Dong,Y., & Simard,J.M. Functional coupling between sulfonylurea receptor type 1 and a nonselective cation channel in reactive astrocytes from adult rat brain. *J. Neurosci.* **23**, 8568-8577 (2003).

51. Soundarapandian,M.M. *et al.* Expression of functional Kir6.1 channels regulates glutamate release at CA3 synapses in generation of epileptic form of seizures. *J. Neurochem.*(2007).

52. Bao,L., Avshalumov,M.V., & Rice,M.E. Partial mitochondrial inhibition causes striatal dopamine release suppression and medium spiny neuron depolarization via H2O2 elevation, not ATP depletion. *J. Neurosci.* **25**, 10029-10040 (2005).

53. Murphy,K.P. & Greenfield,S.A. Neuronal selectivity of ATP-sensitive potassium channels in guinea-pig substantia nigra revealed by responses to anoxia. *J. Physiol* **453**, 167-183 (1992).

54. Matsumoto,N., Komiyama,S., & Akaike,N. Pre- and postsynaptic ATP-sensitive potassium channels during metabolic inhibition of rat hippocampal CA1 neurons. *J. Physiol* **541**, 511-520 (2002).

55. Antcliff,J.F., Haider,S., Proks,P., Sansom,M.S., & Ashcroft,F.M. Functional analysis of a structural model of the ATP-binding site of the KATP channel Kir6.2 subunit. *EMBO J.* **24**, 229-239 (2005).

56. Yamada,M., Ishii,M., Hibino,H., & Kurachi,Y. Mutation in nucleotide-binding domains of sulfonylurea receptor 2 evokes Na-ATP-dependent activation of ATP-sensitive K+ channels: implication for dimerization of nucleotide-binding domains to induce channel opening. *Mol. Pharmacol.* **66**, 807-816 (2004).

57. Matsuo,M., Kimura,Y., & Ueda,K. KATP channel interaction with adenine nucleotides. *J. Mol. Cell Cardiol.* **38**, 907-916 (2005).

58. Seino,S. ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. *Annu. Rev. Physiol* **61**, 337-362 (1999).

59. Baukrowitz,T. *et al.* PIP2 and PIP as determinants for ATP inhibition of KATP channels. *Science* **282**, 1141-1144 (1998).

60. Shyng,S.L. & Nichols,C.G. Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science* **282**, 1138-1141 (1998).

61. Gloyn,A.L. *et al.* Relapsing diabetes can result from moderately activating mutations in KCNJ11. *Hum. Mol. Genet.* **14**, 925-934 (2005).

62. Taschenberger,G. *et al.* Identification of a familial hyperinsulinism-causing mutation in the sulfonylurea receptor 1 that prevents normal trafficking and function of KATP channels. *J. Biol. Chem.* **277**, 17139-17146 (2002).

63. Minami,K., Miki,T., Kadowaki,T., & Seino,S. Roles of ATP-sensitive K+ channels as metabolic sensors: studies of Kir6.x null mice. *Diabetes* **53 Suppl 3**, S176-S180 (2004).

64. Glaser,B. *et al.* Familial hyperinsulinism maps to chromosome 11p14-15.1, 30 cM centromeric to the insulin gene. *Nat. Genet.* **7**, 185-188 (1994).

65. Sagen,J.V. *et al.* Permanent neonatal diabetes due to mutations in KCNJ11 encoding Kir6.2: patient characteristics and initial response to sulfonylurea therapy. *Diabetes* **53**, 2713-2718 (2004).

66. Gloyn,A.L. *et al.* Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit Kir6.2 and permanent neonatal diabetes. *N. Engl. J. Med.* **350**, 1838-1849 (2004).

67. Cartier,E.A., Conti,L.R., Vandenberg,C.A., & Shyng,S.L. Defective trafficking and function of KATP channels caused by a sulfonylurea receptor 1 mutation associated with persistent hyperinsulinemic hypoglycemia of infancy. *Proc. Natl. Acad. Sci. U. S. A* **98**, 2882-2887 (2001).

68. Partridge,C.J., Beech,D.J., & Sivaprasadarao,A. Identification and pharmacological correction of a membrane trafficking defect associated with a

mutation in the sulfonylurea receptor causing familial hyperinsulinism. *J. Biol. Chem.* **276**, 35947-35952 (2001).

69. Nestorowicz,A. *et al.* Genetic heterogeneity in familial hyperinsulinism. *Hum. Mol. Genet.* **7**, 1119-1128 (1998).

70. Shyng,S.L. *et al.* Functional analyses of novel mutations in the sulfonylurea receptor 1 associated with persistent hyperinsulinemic hypoglycemia of infancy. *Diabetes* **47**, 1145-1151 (1998).

71. Miki,T. *et al.* Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. *Proc. Natl. Acad. Sci. U. S. A* **95**, 10402-10406 (1998).

72. Ardehali,H. & O'Rourke,B. Mitochondrial K(ATP) channels in cell survival and death. *J. Mol. Cell Cardiol.* **39**, 7-16 (2005).

73. Hussain,K. & Cosgrove,K.E. From congenital hyperinsulinism to diabetes mellitus: the role of pancreatic beta-cell KATP channels. *Pediatr. Diabetes* **6**, 103-113 (2005).

74. Moreau,C., Prost,A.L., Derand,R., & Vivaudou,M. SUR, ABC proteins targeted by KATP channel openers. *J. Mol. Cell Cardiol.* **38**, 951-963 (2005).

75. Bryan,J. & guilar-Bryan,L. Sulfonylurea receptors: ABC transporters that regulate ATP-sensitive K(+) channels. *Biochim. Biophys. Acta* **1461**, 285-303 (1999).

76. Ardehali,H. Cytoprotective channels in mitochondria. *J. Bioenerg. Biomembr.* **37**, 171-177 (2005).

77. Liu,D., Lu,C., Wan,R., Auyeung,W.W., & Mattson,M.P. Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release. *J. Cereb. Blood Flow Metab* **22**, 431-443 (2002).

78. Gross,G.J. & Fryer,R.M. Sarcolemmal versus mitochondrial ATP-sensitive K+ channels and myocardial preconditioning. *Circ. Res.* **84**, 973-979 (1999).

79. Oldenburg,O., Cohen,M.V., & Downey,J.M. Mitochondrial K(ATP) channels in preconditioning. *J. Mol. Cell Cardiol.* **35**, 569-575 (2003).

80. Shimizu,K. *et al.* MitoK(ATP) opener, diazoxide, reduces neuronal damage after middle cerebral artery occlusion in the rat. *Am. J. Physiol Heart Circ. Physiol* **283**, H1005-H1011 (2002).

81. Seharaseyon,J. *et al.* Molecular composition of mitochondrial ATP-sensitive potassium channels probed by viral Kir gene transfer. *J. Mol. Cell Cardiol.* **32**, 1923-1930 (2000).

82. Cuong,D.V. *et al.* Subunit composition of ATP-sensitive potassium channels in mitochondria of rat hearts. *Mitochondrion.* **5**, 121-133 (2005).

83. Ankarcrona,M. *et al.* Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961-973 (1995).

84. Nicholls,D.G. Mitochondrial dysfunction and glutamate excitotoxicity studied in primary neuronal cultures. *Curr. Mol. Med.* **4**, 149-177 (2004).

85. Gunter,T.E. & Gunter,K.K. Uptake of calcium by mitochondria: transport and possible function. *IUBMB. Life* **52**, 197-204 (2001).

86. Khodorov,B. Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurones. *Prog. Biophys. Mol. Biol.* **86**, 279-351 (2004).

87. Gogvadze,V. & Orrenius,S. Mitochondrial regulation of apoptotic cell death. *Chem. Biol. Interact.* **163**, 4-14 (2006).

88. Lindholm,D., Eriksson,O., & Korhonen,L. Mitochondrial proteins in neuronal degeneration. *Biochem. Biophys. Res. Commun.* **321**, 753-758 (2004).

89. White,R.J. & Reynolds,I.J. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. *J. Neurosci.* **16**, 5688-5697 (1996).

90. Starkov,A.A., Chinopoulos,C., & Fiskum,G. Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. *Cell Calcium* **36**, 257-264 (2004).

91. Michailova,A., Saucerman,J., Belik,M.E., & McCulloch,A.D. Modeling regulation of cardiac KATP and L-type Ca2+ currents by ATP, ADP, and Mg2+. *Biophys. J.* **88**, 2234-2249 (2005).

92. Holmuhamedov,E.L., Wang,L., & Terzic,A. ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria. *J. Physiol* **519 Pt 2**, 347-360 (1999).

93. Wang,L. *et al.* Preconditioning limits mitochondrial Ca(2+) during ischemia in rat hearts: role of K(ATP) channels. *Am. J. Physiol Heart Circ. Physiol* **280**, H2321-H2328 (2001).

94. Kowaltowski,A.J., Castilho,R.F., & Vercesi,A.E. Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca2+ is dependent on mitochondrial-generated reactive oxygen species. *FEBS Lett.* **378**, 150-152 (1996).

95. Kowaltowski,A.J., Netto,L.E., & Vercesi,A.E. The thiol-specific antioxidant enzyme prevents mitochondrial permeability transition. Evidence for the participation of reactive oxygen species in this mechanism. *J. Biol. Chem.* **273**, 12766-12769 (1998).

96. Nicholls,D.G. & Budd,S.L. Mitochondria and neuronal survival. *Physiol Rev.* **80**, 315-360 (2000).

97. Fung,M.L. Role of voltage-gated Na+ channels in hypoxia-induced neuronal injuries. *Clin. Exp. Pharmacol. Physiol* **27**, 569-574 (2000).

98. Steinlein,O.K. & Noebels,J.L. Ion channels and epilepsy in man and mouse. *Curr. Opin. Genet. Dev.* **10**, 286-291 (2000).

99. Ballanyi,K. Protective role of neuronal KATP channels in brain hypoxia. *J. Exp. Biol.* **207**, 3201-3212 (2004).

100. Cuong,D.V. *et al.* Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K+ channels in rat hearts. *Am. J. Physiol Heart Circ. Physiol* **290**, H1808-H1817 (2006).

101. Bancila,V., Nikonenko,I., Dunant,Y., & Bloc,A. Zinc inhibits glutamate release via activation of pre-synaptic K channels and reduces ischaemic damage in rat hippocampus. *J. Neurochem.* **90**, 1243-1250 (2004).

102. Sun,H.S., Feng,Z.P., Miki,T., Seino,S., & French,R.J. Enhanced neuronal damage after ischemic insults in mice lacking Kir6.2-containing ATP-sensitive K+ channels. *J. Neurophysiol.* **95**, 2590-2601 (2006).

103. Plamondon,H., Blondeau,N., Heurteaux,C., & Lazdunski,M. Mutually protective actions of kainic acid epileptic preconditioning and sublethal global ischemia on hippocampal neuronal death: involvement of adenosine A1 receptors and K(ATP) channels. *J. Cereb. Blood Flow Metab* **19**, 1296-1308 (1999).

104. Amoroso,S., Schmid-Antomarchi,H., Fosset,M., & Lazdunski,M. Glucose, sulfonylureas, and neurotransmitter release: role of ATP-sensitive K+ channels. *Science* **247**, 852-854 (1990).

105. Yamada,K. *et al.* Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. *Science* **292**, 1543-1546 (2001).

106. Hernandez-Sanchez,C. *et al.* Mice transgenically overexpressing sulfonylurea receptor 1 in forebrain resist seizure induction and excitotoxic neuron death. *Proc. Natl. Acad. Sci. U. S. A* **98**, 3549-3554 (2001).

107. Yamada,K. & Inagaki,N. Neuroprotection by KATP channels. *J. Mol. Cell Cardiol.* **38**, 945-949 (2005).

108. Ashfield,R., Gribble,F.M., Ashcroft,S.J., & Ashcroft,F.M. Identification of the high-affinity tolbutamide site on the SUR1 subunit of the K(ATP) channel. *Diabetes* **48**, 1341-1347 (1999).

109. Wang,Q., Yu,S., Simonyi,A., Sun,G.Y., & Sun,A.Y. Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol. Neurobiol.* **31**, 3-16 (2005).

110. Gorter,J.A. *et al.* Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca2+ influx in hippocampal CA1 neurons of gerbil. *J. Neurosci.* **17**, 6179-6188 (1997).

111. Mulle,C. *et al.* Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. *Nature* **392**, 601-605 (1998).

112. Perez-Otano,I. & Ehlers,M.D. Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals.* **13**, 175-189 (2004).

113. Wang,J., Liu,S., Fu,Y., Wang,J.H., & Lu,Y. Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. *Nat. Neurosci.* **6**, 1039-1047 (2003).

114. Wang,Y.X., Wenthold,R.J., Ottersen,O.P., & Petralia,R.S. Endbulb synapses in the anteroventral cochlear nucleus express a specific subset of AMPA-type glutamate receptor subunits. *J. Neurosci.* **18**, 1148-1160 (1998).

115. Zhao,H.M., Wenthold,R.J., & Petralia,R.S. Glutamate receptor targeting to synaptic populations on Purkinje cells is developmentally regulated. *J. Neurosci.* **18**, 5517-5528 (1998).

116. Simard,J.M. *et al.* Newly expressed SUR1-regulated NC(Ca-ATP) channel mediates cerebral edema after ischemic stroke. *Nat. Med.* **12**, 433-440 (2006).

117. Nicholls,D. & Attwell,D. The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* **11**, 462-468 (1990).

118. Ozaki,N. *et al.* cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nat. Cell Biol.* **2**, 805-811 (2000).

119. Seino,S. & Shibasaki,T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol Rev.* **85**, 1303-1342 (2005).

120. Shibasaki,T., Sunaga,Y., & Seino,S. Integration of ATP, cAMP, and Ca2+ signals in insulin granule exocytosis. *Diabetes* **53 Suppl 3**, S59-S62 (2004).

121. Rodriguez-Moreno,A. & Lerma,J. Kainate receptor modulation of GABA release involves a metabotropic function. *Neuron* **20**, 1211-1218 (1998).

122. Cho,K. *et al.* Regulation of kainate receptors by protein kinase C and metabotropic glutamate receptors. *J. Physiol* **548**, 723-730 (2003).

123. Melyan,Z., Wheal,H.V., & Lancaster,B. Metabotropic-mediated kainate receptor regulation of IsAHP and excitability in pyramidal cells. *Neuron* **34**, 107-114 (2002).

124. Schmidt-Kastner,R. & Freund,T.F. Selective vulnerability of the hippocampus in brain ischemia. *Neuroscience* **40**, 599-636 (1991).

125. Lipton,P. Ischemic cell death in brain neurons. *Physiol Rev.* **79**, 1431-1568 (1999).

126. Aarts,M. *et al.* Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science* **298**, 846-850 (2002).

127. Aarts,M. *et al.* A key role for TRPM7 channels in anoxic neuronal death. *Cell* **115**, 863-877 (2003).

128. Aarts,M.M. & Tymianski,M. TRPM7 and ischemic CNS injury. *Neuroscientist.* **11**, 116-123 (2005).

129. Huang,Z. *et al.* Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* **265**, 1883-1885 (1994).

130. Iadecola,C., Zhang,F., Casey,R., Nagayama,M., & Ross,M.E. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J. Neurosci.* **17**, 9157-9164 (1997).

131. Kinouchi,H. *et al.* Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. *Proc. Natl. Acad. Sci. U. S. A* **88**, 11158-11162 (1991).

132. Kinouchi,H. *et al.* Role of superoxide dismutase in ischemic brain injury: a study using SOD-1 transgenic mice. *Cell Mol. Neurobiol.* **18**, 609-620 (1998).

133. Bossy-Wetzel,E. *et al.* Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K+ channels. *Neuron* **41**, 351-365 (2004).

134. Fiskum,G., Murphy,A.N., & Beal,M.F. Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. *J. Cereb. Blood Flow Metab* **19**, 351-369 (1999).

135. Krajewski,S. *et al.* Release of caspase-9 from mitochondria during neuronal apoptosis and cerebral ischemia. *Proc. Natl. Acad. Sci. U. S. A* **96**, 5752-5757 (1999).

136. Liu,D. *et al.* Involvement of mitochondrial K+ release and cellular efflux in ischemic and apoptotic neuronal death. *J. Neurochem.* **86**, 966-979 (2003).

137. Lo,E.H., Dalkara,T., & Moskowitz,M.A. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* **4**, 399-415 (2003).

138. Simon,R.P., Swan,J.H., Griffiths,T., & Meldrum,B.S. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226**, 850-852 (1984).

139. Boulter,J. *et al.* Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* **249**, 1033-1037 (1990).

140. Keinanen,K. *et al.* A family of AMPA-selective glutamate receptors. *Science* **249**, 556-560 (1990).

141. Lu,Y.M., Roder,J.C., Davidow,J., & Salter,M.W. Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. *Science* **279**, 1363-1367 (1998).

142. Lu,Y.M., Mansuy,I.M., Kandel,E.R., & Roder,J. Calcineurin-mediated LTD of GABAergic inhibition underlies the increased excitability of CA1 neurons associated with LTP. *Neuron* **26**, 197-205 (2000).

143. Simon,R.P., Swan,J.H., Griffiths,T., & Meldrum,B.S. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226**, 850-852 (1984).

144. Benveniste,H., Drejer,J., Schousboe,A., & Diemer,N.H. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* **43**, 1369-1374 (1984).

145. Drejer,J., Benveniste,H., Diemer,N.H., & Schousboe,A. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. *J. Neurochem.* **45**, 145-151 (1985).

146. Rossi,D.J., Oshima,T., & Attwell,D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* **403**, 316-321 (2000).

147. Choi,D.W. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11**, 465-469 (1988).

148. Choi,D.W. Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci.* **18**, 58-60 (1995).

149. Horn,J. & Limburg,M. Calcium antagonists for ischemic stroke: a systematic review. *Stroke* **32**, 570-576 (2001).

150. Sattler,R. & Tymianski,M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol. Neurobiol.* **24**, 107-129 (2001).

151. Xiong,Z.G. *et al.* Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* **118**, 687-698 (2004).

152. Weiss,J.H., Hartley,D.M., Koh,J., & Choi,D.W. The calcium channel blocker nifedipine attenuates slow excitatory amino acid neurotoxicity. *Science* **247**, 1474-1477 (1990).

153. Goldberg,M.P., Weiss,J.H., Pham,P.C., & Choi,D.W. N-methyl-D-aspartate receptors mediate hypoxic neuronal injury in cortical culture. *J. Pharmacol. Exp. Ther.* **243**, 784-791 (1987).

154. Morikawa,E. *et al.* Attenuation of focal ischemic brain injury in mice deficient in the epsilon1 (NR2A) subunit of NMDA receptor. *J. Neurosci.* **18**, 9727-9732 (1998).

155. Watanabe,M., Mishina,M., & Inoue,Y. Distinct distributions of five NMDA receptor channel subunit mRNAs in the brainstem. *J. Comp Neurol.* **343**, 520-531 (1994).

156. Monyer,H., Burnashev,N., Laurie,D.J., Sakmann,B., & Seeburg,P.H. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**, 529-540 (1994).

157. Burnashev,N., Monyer,H., Seeburg,P.H., & Sakmann,B. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8**, 189-198 (1992).

158. Hollmann,M., Hartley,M., & Heinemann,S. Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science* **252**, 851-853 (1991).

159. Hume,R.I., Dingledine,R., & Heinemann,S.F. Identification of a site in glutamate receptor subunits that controls calcium permeability. *Science* **253**, 1028-1031 (1991).

160. Sommer,B. *et al.* Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* **249**, 1580-1585 (1990).

161. Sommer,B., Kohler,M., Sprengel,R., & Seeburg,P.H. RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* **67**, 11-19 (1991).

162. Verdoorn,T.A., Burnashev,N., Monyer,H., Seeburg,P.H., & Sakmann,B. Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* **252**, 1715-1718 (1991).

163. Oguro,K. *et al.* Knockdown of AMPA receptor GluR2 expression causes delayed neurodegeneration and increases damage by sublethal ischemia in hippocampal CA1 and CA3 neurons. *J. Neurosci.* **19**, 9218-9227 (1999).

164. Pellegrini-Giampietro,D.E., Gorter,J.A., Bennett,M.V., & Zukin,R.S. The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. *Trends Neurosci.* **20**, 464-470 (1997).

165. Tanaka,H., Grooms,S.Y., Bennett,M.V., & Zukin,R.S. The AMPAR subunit GluR2: still front and center-stage. *Brain Res.* **886**, 190-207 (2000).

166. Bonni,A. *et al.* Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-1362 (1999).

167. Ghosh,A., Carnahan,J., & Greenberg,M.E. Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* **263**, 1618-1623 (1994).

168. Mantamadiotis,T. *et al.* Disruption of CREB function in brain leads to neurodegeneration. *Nat. Genet.* **31**, 47-54 (2002).

169. Riccio,A., Ahn,S., Davenport,C.M., Blendy,J.A., & Ginty,D.D. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. *Science* **286**, 2358-2361 (1999).

170. Brene,S. *et al.* Regulation of GluR2 promoter activity by neurotrophic factors via a neuron-restrictive silencer element. *Eur. J. Neurosci.* **12**, 1525-1533 (2000).

171. Shieh,P.B., Hu,S.C., Bobb,K., Timmusk,T., & Ghosh,A. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* **20**, 727-740 (1998).

172. Tabuchi,A., Sakaya,H., Kisukeda,T., Fushiki,H., & Tsuda,M. Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. *J. Biol. Chem.* **277**, 35920-35931 (2002).

173. Calderone,A. *et al.* Ischemic insults derepress the gene silencer REST in neurons destined to die. *J. Neurosci.* **23**, 2112-2121 (2003).

174. Higuchi,M. *et al.* Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. *Nature* **406**, 78-81 (2000).

175. Liu,S.Q. & Cull-Candy,S.G. Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* **405**, 454-458 (2000).

176. Rozov,A. & Burnashev,N. Polyamine-dependent facilitation of postsynaptic AMPA receptors counteracts paired-pulse depression. *Nature* **401**, 594-598 (1999).

177. Anzai,T. *et al.* Overexpression of Ca2+-permeable AMPA receptor promotes delayed cell death of hippocampal CA1 neurons following transient forebrain ischemia. *Neurosci. Res.* **46**, 41-51 (2003).

178. Jia,Y., Jeng,J.M., Sensi,S.L., & Weiss,J.H. Zn2+ currents are mediated by calcium-permeable AMPA/kainate channels in cultured murine hippocampal neurones. *J. Physiol* **543**, 35-48 (2002).

179. Sensi,S.L., Yin,H.Z., Carriedo,S.G., Rao,S.S., & Weiss,J.H. Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. *Proc. Natl. Acad. Sci. U. S. A* **96**, 2414-2419 (1999).

180. Weiss,J.H., Hartley,D.M., Koh,J.Y., & Choi,D.W. AMPA receptor activation potentiates zinc neurotoxicity. *Neuron* **10**, 43-49 (1993).

181. Weiss,J.H. & Sensi,S.L. Ca2+-Zn2+ permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. *Trends Neurosci.* **23**, 365-371 (2000).

182. Yin,H.Z., Sensi,S.L., Ogoshi,F., & Weiss,J.H. Blockade of Ca2+-permeable AMPA/kainate channels decreases oxygen-glucose deprivation-induced Zn2+ accumulation and neuronal loss in hippocampal pyramidal neurons. *J. Neurosci.* **22**, 1273-1279 (2002).

183. Koh,J.Y. *et al.* The role of zinc in selective neuronal death after transient global cerebral ischemia. *Science* **272**, 1013-1016 (1996).

184. Shi,S., Hayashi,Y., Esteban,J.A., & Malinow,R. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105**, 331-343 (2001).

185. Bredt,D.S. & Nicoll,R.A. AMPA receptor trafficking at excitatory synapses. *Neuron* **40**, 361-379 (2003).

186. Bredt,D.S. & Nicoll,R.A. AMPA receptor trafficking at excitatory synapses. *Neuron* **40**, 361-379 (2003).

187. Jensen,M.S., Lambert,J.D., & Johansen,F.F. Electrophysiological recordings from rat hippocampus slices following in vivo brain ischemia. *Brain Res.* **554**, 166-175 (1991).

188. Xu,Z.C. & Pulsinelli,W.A. Electrophysiological changes of CA1 pyramidal neurons following transient forebrain ischemia: an in vivo intracellular recording and staining study. *J. Neurophysiol.* **76**, 1689-1697 (1996).

189. Hayashi,Y. *et al.* Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287**, 2262-2267 (2000).

190. Rogers,S.W. *et al.* The characterization and localization of the glutamate receptor subunit GluR1 in the rat brain. *J. Neurosci.* **11**, 2713-2724 (1991).

191. Wenthold,R.J., Petralia,R.S., Blahos,J., II, & Niedzielski,A.S. Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J. Neurosci.* **16**, 1982-1989 (1996).

192. Greger,I.H., Khatri,L., & Ziff,E.B. RNA editing at arg607 controls AMPA receptor exit from the endoplasmic reticulum. *Neuron* **34**, 759-772 (2002).

193. Greger,I.H., Khatri,L., Kong,X., & Ziff,E.B. AMPA receptor tetramerization is mediated by Q/R editing. *Neuron* **40**, 763-774 (2003).

194. Kawahara,Y. *et al.* Glutamate receptors: RNA editing and death of motor neurons. *Nature* **427**, 801 (2004).

195. Van,D.P., Van Den,B.L., Van,H.E., Callewaert,G., & Robberecht,W. GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *J. Neurophysiol.* **88**, 1279-1287 (2002).

196. Ikonomidou,C. & Turski,L. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol.* **1**, 383-386 (2002).

197. Morris,G.F. *et al.* Failure of the competitive N-methyl-D-aspartate antagonist Selfotel (CGS 19755) in the treatment of severe head injury: results of two phase III clinical trials. The Selfotel Investigators. *J. Neurosurg.* **91**, 737-743 (1999).